TCR γδ Bearing Lymphocyte Clones With Lymphokine-Activated Killer Activity Against Autologous Leukemic Cells

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Activated T lymphocytes with the T-cell receptor (TCR) γδ (CD3⁺ and TCR δ⁺) exhibit strong cytotoxic activity against the standard natural killer (NK) and lymphokine-activated killer (LAK) sensitive target cells. In order to test the cytotoxic activity of γδ T lymphocytes against autologous leukemic cells, 84 clones of γδ T lymphocytes were obtained from the peripheral blood of three acute lymphoblastic leukemia (ALL) patients. Forty-four of these T-cell clones were active against an LAK-sensitive cell line and the other 40 were active against K562, an NK target cell line. In each of the three patients, cytotoxic clones against autologous leukemic cells were obtained. Among the 84 clones, ten were able to kill autologous tumor cells, including eight that lyse the LAK-sensitive target and two with NK activity. The clones were highly cytotoxic, stable, and easily expanded in large quantity.

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IMMUNOTHERAPY with in vitro IL-2 lymphokine-activated killer (LAK) cells and systemic recombinant IL-2 resulted in marked tumor regression in some solid-tumor patients for whom there was no other effective therapy. LAK cells are generally defined by their in vitro MHC-unrestricted cytotoxic activity against a panel of cultured leukemic cells. However, an LAK activity is also seen against fresh leukemic cells. Previous studies have reported that LAK cells are derived from blood natural killer (NK) cell population of large granular lymphocytes (LGLs), which do not express the CD3 molecules. In addition, recent evidence suggests that CD3-bearing lymphocytes with cytotoxic activity against autologous tumor target predominate in tumor infiltrating lymphocytes (TIL) from metastatic melanoma. CD3⁺ T lymphocytes are subdivided into two subsets according to the type of lymphocyte T-antigen receptor (TCR). Most of the CD3⁺ cells in the peripheral blood of normal individuals exhibit the αβ heterodimer that recognizes antigen in an MHC-restricted fashion. These cells react with the WT31 monoclonal antibody (specific for a framework determinant of the TCR αβ), and express CD4 or CD8 (molecules associated with class II or class I MHC antigen recognition). Only a small cell subset initially identified by their lack of reactivity with the WT31 monoclonal antibody bears a γδ TCR. Once activated, some of these T lymphocytes perform a strong cytotoxic activity against the standard NK- and LAK-sensitive target cells. Since more than half of circulating γδ T lymphocytes do not express the CD8 molecule, it is tempting to postulate that their cytolytic activity is non-MHC-restricted and corresponds to an NK-like or LAK activity.

The main objective of our study was to determine if TCR γδ-expressing T-lymphocyte clones derived from adult acute leukemia patients in complete remission (CR) were capable of exhibiting cytotoxic activity against autologous leukemic cells and maintaining the same pattern of reactivity after being expanded in vitro over 10⁶ cells. This was done in order to obtain a pure population of cytotoxic cells active against autologous tumor cells, which is easily expandable in large quantity for treatment of the patients in subsequent relapse. We report the results of a study using autologous cell clones of TCR γδ-bearing T lymphocyte with cytolytic activity in three cases of acute lymphoblastic leukemia.

MATERIALS AND METHODS

Patients and cell preparation. Three adult patients (Mo, Le, and Ro) with acute lymphoblastic leukemia (ALL) were treated at Hôpital St Louis, Paris. When CR was achieved, 50 × 10⁶ peripheral blood mononuclear cells (PBMC) from 50 mL of blood were obtained by Ficoll-Hypaque density gradient centrifugation. The characteristics of blast cells are presented in Table 1. Blast cells were recovered before treatment and cryopreserved for use as target cells in subsequent cytotoxic tests.

Generation of cytotoxic T-cell clones. PBMC containing 2% to 30% of CD3⁺ WT31⁺ cells were stained with a mixture of CD4 and CD8 monoclonal antibodies (MoAbs) followed by treatment with rabbit complement for one hour at 37°C. Viable cells were cultured under limiting conditions in the presence of irradiated allogeneic PBMC as previously described. One microgram per milliliter of PHA (Wellcome, Beckenham, UK) and 25 U/mL of recombinant IL-2 (Roussel Uclaf, Romainville, France) were added to the culture medium. The culture medium used was RPMI 1640 (GIBCO, Paisley, UK) supplemented with 10% heat-inactivated human AB serum, with 2 mmol/L L-glutamine and penicillin (100 U/mL) streptomycin (100 μg/mL). Clones were screened directly by FACS analysis (Facstar, Becton Dickinson, Mountain View, CA) for the presence of surface-CD3 TCR γδ and the simultaneous lack of CD4, CD8, and TCR αβ. The MoAbs used in these experiments were OKT3, OKT4, OKT8, WT31 and anti–TCR δ-1. Anti-TCR δ is a murine MoAb reactive with a framework epitope on the γδ TCR (ascitis was generously provided by Dr M. Brenner, DFCI, Boston). The CD3⁺, TCR-δ⁺, WT31⁺, CD4⁻, and CD8⁻ clones were stimulated every 2 weeks with PHA, rIL-2, and irradiated feeder cells in flasks (Costar, Cambridge, MA).

Phenotypic analysis of cell surface antigens. Phenotypic analysis was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse F(ab')₂ IgG. Samples were analyzed on a cytofluorograph (Facstar, Becton Dickinson) in each sample, 10⁴ cells were analyzed. Ascities derived from a nonreactive hybridoma was used as negative control to determine background fluorescence.

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Monoclonal antibodies were always used at saturating concentrations (1:100 to 1:1,000). The reactivity of OKT3 and anti-TCR γδ has been stable in at least five determinations over a 2-month period of time. The phenotype of nine representative cell clones with both monoclonal antibodies is shown in Fig 1.

**Cytotoxicity assays.** Cytotoxicity assays were performed according to a standard 51Cr-release method described previously. All experiments were performed on day 8 of the 14-day restimulation culture. Before testing, the cloned cells were washed once and then left overnight in 10% human AB serum-RPMI medium (2 x 10^6 cells/mL). Assays with 5 x 10^5 51Cr-labeled target cells per well were done in triplicate, using V-bottomed microtiter plates (Greiner, Nürtingen, FRG). Fresh leukemia blasts were treated with 10 μg/mL of DNase (Boehringer Mannheim, FRG) for two hours during the labeling (100 μCi of 51Cr).

**Target cells for NK and LAK assays.** Cultured K562 and Daudi cell lines, plus thawed cryopreserved blasts were employed. K562 is a myeloid cell line established from a patient with chronic myelogenous leukemia, and is sensitive to both NK and LAK killing, while Daudi is a Burkitt’s lymphoma cell line and is largely NK-resistant but LAK-sensitive. Both cell lines were mycoplasma-free and maintained in logarithmic growth in RPMI-1640 containing 15% fetal calf serum and antibiotics.

**RESULTS**

**Heterogeneous cytotoxic reactivity expressed by the CD3+ γδ TCR-bearing cell clones.** Eighty-four γδ TCR clones of T lymphocytes were obtained from three patients (Table 2) and analyzed for their cytotoxic activity against various target cells. Cultured under our experimental conditions, all T-cell clones mediated strong cytotoxic activity. The data of cell-mediated lysis at a 2:1 effector to target (E:T) ratio presented in Table 2 show a diversity of lytic specificity within the T-cell clones when tested against the NK-sensitive K562 cell line and LAK-sensitive Daudi cell line. The cytotoxic activity was verified at various E:T ratios, and in all experiments the decrease of the percent of specific lysis followed the decrease in E:T ratios (data not shown). Further studies were done in order to determine the cytolytic activity against autologous tumor cells. The results show (Table 2) that it is possible to isolate and culture γδ TCR-expressing cells with anti-autologous tumor killer activity from the peripheral blood of remission patients studied. This cytotoxic activity against the autologous leukemic cells is found predominantly in the subset of cells that lyse the Daudi cell line (8/10). However, the majority of clones that mediate reactivity against the LAK-sensitive cell line are not active against the autologous tumor cells (36/44). Few NK-like cells are capable of killing the autologous tumor cells (2/40). These T-cell clones never exhibited cytotoxic activity against three days’ autologous PHA-blasts. In contrast, fresh allogeneic leukemic cells were killed by the clones that lysed the Daudi cell line. Individual clones were seen to have different repertoires for lysis (data not shown). These clones maintained stable cytolytic activity, phenotype,
and cytotype (tested after 6 months). The cell growth is dependent on IL-2 and feeder cells.

DISCUSSION

Peripheral blood lymphocytes with cytolytic activity are mainly subdivided in two subpopulations: T lymphocytes reacting with the CD3 monoclonal antibodies, and non-T lymphocytes that are CD3-. LAK activity defined by a non-MHC-restricted cytotoxic activity toward tumor cell lines such as the Daudi cell line, is found among cells from both subpopulations. In the CD3+ population, cells with NK cytolytic activity possessing CD16 cell surface markers are good candidates for LAK activity. Highly purified CD16 cell subsets could be proposed to be LAK cells for the treatment of patients with malignancies. However, these cells expanded in our culture conditions do not warrant a perfect functional stability of the subpopulation (A. Bensus-
an, unpublished observation).

CD3+ T lymphocytes can be cloned. We have previously reported that a non-MHC-restricted cytotoxic activity toward the Daudi cell line is found in the CD4+CD8- γδ TCR subset.13 The CD8+ αβ TCR subset could also be cytolytic against tumor cells, but specifically and through an MHC restriction corresponding to the TIL.14

In order to obtain cells with LAK activity at the clonal level, we focused our study on γδ TCR+ CD4-/CD8- γδ T lymphocytes. The question of relationship between the so-called LAK activity and the ability to lyse autologous tumor cells was solved by the choice of using malignant cells from ALL adult patients as target cells. Three patients, two of them with poor prognosis (Mo and Le), were initially studied. The malignant cells did not express IL-2 receptor (CD25-).

Among 84 γδ TCR cell clones, only ten mediated an anti-autologous tumor cell cytolytic activity. In each of the three patients, γδ TCR cell clones with anti-autologous tumor cytolytic activity were obtained. Eight are LAK cells and two are NK-like cells. They represent a minority of the LAK (8/44) and NK-like (2/40) γδ TCR cell clones. Thus, LAK activity is not always associated to a cytotoxicity against autologous leukemic target cells.

Thus, in the peripheral blood of ALL complete remission patients studied, between 2% and 7% of CD3+ T lymphocytes are γδ TCR. Once activated, 50% of these cells are LAK cells and only 18% of these LAK cells are cytotoxic against the autologous tumor cells. These γδ clones are easily expanded in vitro (>2106 cells/week for each clone) and their cytolytic activity is very strong compared to the usual LAK cells, as one effector cell is able to kill ten K562 target cells.

Taking all these results into account, the γδ TCR cell clones could be used as therapeutic agents against ALL tumor cells, either to purify bone marrow before autologous graft or to consolidate complete remission in patients with poor prognosis. Moreover, in vivo infusion could give information on the homing and survival of the clones and its descendant clones. These cells can be easily detected using clone-specific oligonucleotide probes directed against Vγ-Jγ or Vδ-Jδ junctional sequences after PCR amplification.20 No gene transfer is needed by this method, and precise results restricted to the population active against the autologous tumor cells will be available.

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