Characterization of Natural Killer Cells With Antileukemia Activity Following Allogeneic Bone Marrow Transplantation

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To identify cells with potential antileukemia activity following bone marrow transplantation, we have monitored immunologic reconstitution in a patient with acute lymphocytic leukemia in second remission who received intensive chemotherapy and total body irradiation followed by infusion of allogeneic histocompatible marrow. Prior to transplantation, donor bone marrow cells were depleted of T lymphocytes by in vitro treatment with anti-T12 monoclonal antibody and rabbit complement. In the first 3 weeks following bone marrow transplantation, the predominant regenerating mononuclear cell population in peripheral blood exhibited a phenotype characteristic of natural killer (NK) cells. After 4 weeks, T lymphocytes became predominant, but NK cells persisted. Cultured peripheral blood lymphocytes obtained 12 weeks posttransplant were able to display significant cytoxicity against leukemic blasts that had been cryopreserved at the time of relapse 5 months prior to bone marrow transplantation. To further characterize those cells with antileukemia activity, we

Within the last 10 years intensive chemotherapy and total body irradiation (TBI) followed by transplantation of allogeneic bone marrow has become established as an effective and potentially curative therapeutic modality for patients with various forms of leukemia. In this combined modality therapy, it is widely assumed that eradication of leukemia is primarily accomplished by high-dose chemotherapy and TBI and that the transplantation of allogeneic marrow simply acts in a supportive role to reconstitute marrow function that has been irreversibly damaged by the ablative treatment program. In addition, it has been postulated that immunologic reconstitution with allogeneic cells may in itself have a significant antileukemic effect. This view is supported by the recent finding that those transplant recipients in whom significant graft-versus-host disease (GVHD) developed have a decreased risk of leukemia relapse compared to patients in whom GVHD does not develop. The present studies were undertaken to investigate the possible existence of cells with direct cytoxicity for leukemic cells following bone marrow transplantation (BMT) and to determine whether this activity could be distinguished from GVHD. In so doing, we undertook phenotypic and functional characterization of peripheral blood lymphocytes (PBL) regenerating following allogeneic BMT in a single patient with T cell acute lymphoblastic leukemia (T-ALL) from whom we had been able to cryopreserve leukemic blasts at the time of relapse 5 months prior to transplantation. Using recently developed methods for establishing and expanding cloned cytotoxic cells without preselected specificity, we identified four distinct clonal cell lines capable of mediating cytoxicity against the patient’s leukemic blasts. Further characterization of these cloned cell lines demonstrated that all exhibited the phenotype of natural killer (NK) cells as well as functional NK activity.

Materials and Methods

Monoclonal antibodies. All monoclonal antibodies used in this study have been described previously. Briefly, anti-T3 defines all mature T lymphocytes. T4 antigen is expressed on a subset of peripheral T cells having primarily inducer function. T8 antigen is expressed on a subset of peripheral T cells having primarily cytotoxic/suppressor function. Anti-T11 defines an antigen associated with the sheep erythrocyte receptor. T12 antigen is expressed on a fraction of thymocytes and the vast majority of peripheral T cells. Anti-NKH1 defines a pan-NK cell antigen that is expressed on all resting as well as activated NK cells and NK clones. Anti-NKH2 characterizes a subset of large granular lymphocytes (LGL) in peripheral blood and is only expressed on some NK active clones. Anti-B1 reacts with all normal B cells, and anti-MY4 reacts with all peripheral blood monocytes. Anti-T3, T4, T8, T11, T12, B1, MY4, and NKH1 antibodies are available from Coulter Immunology, Hialeah, Fla.

Phenotypic analysis of cell surface antigens. Phenotypic analysis was performed by indirect immunofluorescence with fluorescein-
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Samples were analyzed on an Epics V (Coulter Electronics, Hialeah, Fla) or a FACS 440 (Becton Dickinson, Mountain View, Calif) flow cytometer. Ten thousand cells were analyzed in each sample. Negative controls used to determine background fluorescence were ascites derived from a nonreactive hybridoma. Monoclonal antibodies were always used at saturating concentrations (1:500 to 1:1,000).

Cytotoxicity assays. Cytotoxicity assays were performed according to a standard chromium release method previously described. All experiments were done in triplicate using V-bottom microtiter plates. Cytotoxicity assays were performed usually for three hours at 37°C using 5,000 target cells/well. Medium was RPMI 1640 plus 5% pooled human AB serum and 1% penicillin-streptomycin. Target cells in these assays included Molt-4 and JM, which are T cell leukemia cell lines. K562 was derived from a patient with chronic myelogenous leukemia. U937 is a histiocytic cell line. Lazu909 is an Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell line.

Preparation of lymphocyte-conditioned media. Lymphocyte-conditioned medium (LCM) was produced by stimulation of peripheral mononuclear cells (PBMC) at a concentration of 2 x 10^6 with 4 μg/mL phytohemagglutinin (Burroughs Wellcome, Greenville, NC), 5 μg/mL phorbol myristate acetate; (Sigma Chemical Co, St Louis), and irradiated (5,000 rad) EBV-transformed B lymphoblastoid cells (PBMC/irradiated EBV-transformed B cells ratio, 3:1). Following a first incubation for three hours at 37°C, cells were washed four times to remove the mitogens and resuspended in RPMI 1640 supplemented with 2.5% human AB serum. After 40 hours of subsequent incubation at 37°C, culture supernatants were harvested, passed through 0.22-μm filters and stored at -70°C.

Generation of cloned cell lines. Methods for generation of human NK cloned cell lines have been previously described in detail. Briefly, clones were obtained using a limiting-dilution technique at 0.5 cells/well. Cell lines were generated using the same experimental conditions, but cloning was done at 100 colonies in each well.

Cytogetic analysis. Bone marrow cells from the patient obtained prior to transplantation were cultured for 24 hours at 37°C. Cells were first treated with 0.1 μg/mL Colcemid (GIBCO, Grand Island, NY) for ten minutes and then with 75 mmol/L KCl for 20 minutes at 37°C. Following hypotonic treatment, cells were centrifuged and the cell pellets broken up and fixed in 3:1 (vol/vol) methanol/acetic acid for one hour. After two changes of fixative, standard air-dried slides were prepared. Peripheral blood cultures from the donor were set up in media containing PHA, incubated for 72 hours at 37°C, and processed as previously described. Cultured cell lines were treated with 0.01 μg/mL Colcemid for 30 minutes, treated with hypotonic KCl for ten minutes at 37°C, and processed as previously described. All slides were stained with quinacrine mustard (25 μg/mL), rinsed in running tap water, and mounted in Tris-malate buffer, pH 5.6. Fluorescence-banding patterns were recorded on Kodak Technicolor Pan film using a Leitz Ortholux fluorescence microscope equipped with an Orthomath camera.

CASE REPORT

This 32-year-old male was initially found to have acute lymphocytic leukemia in Dec 1980. At that time his primary complaint was chest pain and dyspnea, and chest x-ray results demonstrated a large anterior mediastinal mass. An examination of the bone marrow demonstrated a replacement of normal marrow cells with a diffuse infiltrate of lymphoblasts. Remission induction was achieved following the administration of Adriamycin, vincristine, prednisone, and L-asparaginase. He subsequently received CNS irradiation and intrathecal methotrexate and maintenance chemotherapy with intermittent cycles of prednisone, vincristine, methotrexate, and 6-mercaptothiazole. Adriamycin was added to the maintenance regime every 3 months. After 36 months of therapy, examination of the peripheral blood and bone marrow demonstrated a leukemia relapse. The peripheral WBC count was 120,000/μL, with almost all cells being lymphoblasts. The bone marrow was markedly hypercellular and was again almost totally replaced with lymphoblasts. The phenotype of bone marrow lymphoblasts as determined by indirect immunofluorescence assay and cell sorter analysis was as follows: T11+, T6-, T3 weakly +, B4-, B1-, common acute lymphoblastic leukemia antigen-negative, MY9-. Further treatment with vincristine, prednisone, Adriamycin, and L-asparaginase was able to induce a second remission.

While in second remission, he underwent allogeneic marrow transplantation with cells from his histocompatible brother (HLA-A, B, C, D) identical and mixed lymphocyte culture nonreactive). Prior to infusion of marrow he received intensive therapy consisting of the following: cytosine arabinosine, 3 g/m² intravenously (IV) (four doses on days -5 and -4), cyclophosphamide, 60 mg/kg IV (two doses on days -5 and -4), TBI, 1,200 rad (200-rad fractions twice daily on days -3, -2, and -1). To prevent GVHD, donor marrow was treated in vitro with anti-T12 monoclonal antibody and rabbit complement to remove mature T cells. The total number of infused cells was 2.2 x 10^8. No other GVHD prophylaxis was administered. This protocol was approved by the Institutional Review Boards of the Dana-Farber Cancer Institute and Brigham and Women's Hospital. Before informed consent was obtained, the procedure and potential risks and benefits were explained to the patient and his family.

Hematopoietic reconstitution posttransplantation proceeded without complications. Nucleated RBCs were first seen in peripheral blood on day 8. Circulating granulocytes rose to levels >500/μL by day 17. The platelet count remained >20,000 without platelet transfusions after day 14. By day 19 posttransplant, the WBC count was 2,600/μL with 42% granulocytes, the hematocrit value was 37%, and the platelet count was 38,000/μL. Engraftment with allogeneic cells of donor origin was documented by cytogetic analysis that identified chromosomal polymorphisms of donor type in metaphase preparations of marrow and stimulated peripheral blood lymphocytes posttransplantation. Acquisition of the donor-type red cell antigen phenotype was also documented posttransplant. He was discharged on day 20 after marrow transplantation. Following discharge, his clinical course was complicated by the development of right upper-quadrant abdominal pain, ascites, pedal edema, and a mildly elevated bilirubin level (total, 2.0 mg/dL). This was felt to be secondary to veno-occlusive disease of the liver and responded rapidly to treatment with oral prednisone (40 mg/d). During his early posttransplant course, mild skin erythema developed that resolved without specific treatment by day 20. Diarrhea or other manifestations of GVHD never developed. At present, he continues in remission 20 months posttransplant.

RESULTS

Phenotypic analysis of PBMC posttransplantation. At frequent intervals posttransplantation, PBMC were obtained and analyzed for reactivity with a series of monoclonal antibodies defining antigens expressed by normal T cells, B cells, and NK cells.
cells, NK cells, and monocytes. Results of this analysis on days 11, 18, 29, and 58 post-BMT are shown in Fig 1. In the first 3 weeks post-BMT, cells expressing NKH1A represented the major population of early reconstituting mononuclear cells. Two-color immunofluorescence assays demonstrated that the NKH1A-positive cells also expressed T11/E rosette antigen but did not express T3 antigen (data not shown). Although a minor population of mature T cells expressing T3, T11, and T4 or T8 antigens were also present during this time, there were very few cells expressing T12 antigen, which is also found on normal mature T cells in peripheral blood. By 4 weeks post-BMT, the percentage of NKH1A-positive cells had decreased, and the percentage of cells expressing T3, T11, and T4 or T8 antigens had increased so that T lymphocytes now represented the major population of PBMC. At this time, T cells were also found to express T12 antigen and thus displayed a more normal pattern of T cell surface markers. A high level of NK activity was maintained even after T lymphocytes became the predominant mononuclear cell in peripheral blood. When tested against K562 target cells, uncultured PBMC obtained 34 days post-BMT exhibited a 48% specific cytotoxicity at 60:1 effector/target cell (E/T) ratio, 35% at 30:1, and 22% at 15:1.

Circulating B cells identified by reactivity with anti-B1 first appeared 9 weeks post-BMT. A moderate degree of monocytosis was evident by reactivity with anti-MY4 on days 18 and 29 but did not persist after 4 weeks. During the first 3 months post-BMT, cells expressing the Ia antigen consistently represented >50% of PBMC (data not shown), suggesting that these cells were activated and proliferating in vivo. Expression of the NKH2 antigen varied between 10% and 29% of PBMC.

Generation of polyclonal lymphocytic cell lines pre- and post-allogeneic marrow transplantation. In conjunction with the phenotypic and functional characterization of lymphocyte reconstitution post-BMT, polyclonal lymphoid cell lines were established from peripheral blood obtained before and after allogeneic transplantation using in vitro stimulation and subsequent expansion with interleukin 2 (IL 2). The lymphoid cell line developed from pretransplant cells (termed TL-pre) was generated from a vial of PBMC cryopreserved at the time of leukemic relapse, 5 months prior to transplantation. Generation of this cell line was based on the assumption that a small percentage of normal T lymphocytes was still present in the peripheral blood during the acute phase of the disease. Thawed cells were stimulated with concanavalin A and cultured for 2 weeks in the presence of LCM containing IL 2. After 2 weeks, leukemic blasts were no longer detectable in the culture. Remaining viable cells were then expanded by plating 100 cells/well onto a feeder layer of irradiated normal PBL and EBV-transformed B cell line as described in Materials and Methods. The phenotype of this cell line is summarized in Table 1. TL-pre cells were primarily mature T lymphocytes expressing T3, T11, and T12 antigens. This cell line contained approximately equal percentages of T4+ (37%) and T8+(32%) cells and relatively small percentages of cells expressing NKH1A (13%) and NKH2 (15%) antigens. This phenotype was clearly different from that of the T-ALL blasts, which expressed T3 antigen only weakly and did not express either T4 or T8 antigens.

The posttransplant cell line (termed TL-post) was established from peripheral blood obtained 83 days post-BMT and was subsequently used to develop cloned cell populations for the identification of cells with antileukemic activity. TL-post was obtained following stimulation and expansion of PBMC in LCM until sufficient numbers of cells were available for phenotypic and functional characterization. The phenotype of the TL-post cell line is summarized in Table 1. The great majority of these cells expressed T11 antigen, but in contrast to TL-pre cells, fewer cells were T3+, almost all cells were T8+, and very few were T4+. Expression of T12 antigen was less than expression of T3 antigen and was considerably weaker than in TL-pre cells. In addition, a greater percentage of the TL-post lymphocytes expressed the NK cell markers NKH1A (47%) and NKH2 (35%). More importantly, TL-post lymphocytes displayed significant activity against the patient's cryopreserved leukemic cells (23% specific cytotoxicity at 60:1 E/T ratio).

Generation of cloned cell lines displaying antileukemic activity. Cells obtained from the polyclonal TL-post cell line were cloned at 0.5 cells/well using procedures described in Materials and Methods. Clonal expansion was maintained by the addition of LCM every three days. Between 2 and 3 weeks following cloning, 74 macroscopic colonies were apparent (cloning efficiency, 30%). Cloned colonies were individually screened for cytotoxicity against uncultured freshly thawed leukemic blasts in a conventional four-hour 51Cr release assay. Of the 74 colonies, six displayed cytotoxicity against the leukemic blasts. These six colonies were...
The TC clones were also tested against normal activated T lymphocytes derived from peripheral blood cells of the patient. For this purpose, lymphocytes from the TL-pre cell line were used as target cells. It was found that TC12, -48, -50, and -59 had no cytotoxic activity against normal activated T lymphocytes autologous to the T cell leukemic blasts (Table 2).

**Phenotypic analysis of clones TC12, -48, -50, and -59.** Phenotypic analysis of antileukemic clones was performed using indirect immunofluorescence assays and subsequent analysis by flow cytometry. The clones were tested with a series of monoclonal antibodies that have been previously used to characterize NK cloned cell lines derived from normal donors and are included for comparison in Table 3. TC48 cells were found to express NKH1α antigen but did not react with anti-T3, -T4, -T8, or -T11. This pattern of reactivity appeared to be similar to that of a previously described clone termed JT1.8,14 However, as opposed to JT1 clones, TC48 cells did not express NKH2 antigen. It was also found that TC48 cells did not react with anti-T11 and anti-T11 antibodies, which define other epitopes of the E rosette receptor molecule18 (data not shown). Clone TC59 was found to express T11 as well as NKH1α and NKH2 antigens, a phenotype similar to that of the cloned NK cell line JT3.14 Clones TC12 and TC50 displayed an identical phenotype using this series of antibodies. These two latter clones were found to express T11 and NKH1α but did not react with anti-NKH2 and were thus similar to the NK clone JT3.18 None of the clones expressed the T cell antigens T3, T4, or T8.

**Cytogenetic characterization of clones TC12, -48, -50, and -59.** Quinacrine bright polymorphisms were compared between metaphases obtained from the patient’s bone marrow cultures and PHA-stimulated leukocyte cultures from the donor. One informative polymorphism was observed. Although both copies of chromosome 21 from the patient lacked any polymorphism (Fig 3A), one of the copies of chromosome 21 in the donor had quinacrine bright satellites (Fig 3B). Twenty-seven Q-banded metaphases from the four clones were analyzed. All of them had a chromosome 21 with polymorphic satellites; the clones expressed the T cell antigens T3, T4, or T8.

**DISCUSSION**

In the present study, peripheral blood cells from a single individual were analyzed at various times during immunologic reconstitution following allogeneic BMT with T cell-depleted marrow. The primary goal of this characterization was to identify those cells that might be capable of exerting a

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**Table 2. Cytoxicity of Antileukemic Clones Against Conventional NK Target Cell Lines and Normal Activated T Cells**

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*Percent specific cytotoxicity, mean of triplicate assays. SD ≤5%.*/
Table 3. Antigenic Phenotype of Cloned Cell Lines Mediating Antileukemic Activity: Comparison With NK Clones Derived From Normal Donors

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<tr>
<th>Clone</th>
<th>T3</th>
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<th>NKH2</th>
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<tr>
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<td>TC50</td>
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Key: - , <5% of cells reactive; + , >90% of cells reactive.
*Expression of antigens determined by indirect immunofluorescence reactivity with specific monoclonal antibodies.
†Derived from normal donor.

direct cytotoxic effect against the patient's leukemic blasts. In this regard, we found that the major population of PBMC reconstituting during the first 3 weeks post-BMT had an NK cell phenotype and exhibited high levels of NK activity. Only after 1 month did large numbers of T cells develop. At 12 weeks post-BMT, the peripheral blood still contained 24% NKH1<sub>a</sub>-positive cells, and a polyclonal cell line established at this time was able to mediate a relatively low but significant level of cytotoxic activity against freshly thawed leukemic cells that had been cryopreserved at the time of relapse. To identify the cells responsible for this antileukemic activity, cultured cells were cloned at 0.5 cells/well using techniques that had previously been developed to establish clonal populations of NK-active cells from normal individuals. Using these techniques, four different clonal cell lines with antileukemic activity could be identified and propa-
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gated in sufficient numbers to allow a detailed phenotypic and functional characterization. Cytogenetic analysis confirmed that each clone was derived from the HLA-compatible allogeneic donor. Immunofluorescence analysis of cell surface markers demonstrated that each clone had a characteristic phenotype representative of NK cells and that these phenotypes were similar to those of NK clones previously derived from normal individuals. Finally, each clone was cytotoxic for standard NK target cells as well as for leukemic blasts, but these clones did not exhibit cytotoxicity for normal activated T cells derived from the patient prior to BMT.

Taken together, the clonal analysis of cytotoxic cells in our patient suggests that activated NK cells derived from the donor may be capable of exerting a direct antileukemic effect following allogeneic marrow transplantation. This conclusion is consistent with the results of experiments in animal models that have suggested that NK cells play a role in normal immune resistance to radiation-induced leukemias as well as other hematopoietic and nonhematopoietic tumors. In the context of marrow transplantation, however, NK cells have been implicated in a number of sometimes conflicting responses. For example, several studies have demonstrated that NK cells in the host can mediate immunologic resistance to an allogeneic marrow graft. It has also been suggested that NK cells in the allogeneic graft play a role in producing GVHD, whereas other studies have found no correlation between NK activity post-BMT and either leukemia relapse or GVHD.

The implication of NK cells in such diverse activities may reflect the extensive phenotypic and functional heterogeneity of these cells. In previous studies, we and others have established cloned cell lines that maintain NK activity from normal peripheral blood and have used these cultured cells to characterize NK activity at the single-cell level. The diversity of phenotypes and functional specificity demonstrated with these cloned NK cell lines appeared to reflect the heterogeneity of uncultured cells capable of mediating NK function, and several of these cloned cell lines were used for comparative purposes in the present analysis. Of interest, several normal NK clones were either not able to kill our patient's cryopreserved leukemic cells or had very low levels of activity even though their phenotype and cytotoxicity for other NK targets was similar to that seen with clones TC12, -48, -50, and -59. Moreover, it should also be pointed out that the ability to kill leukemic targets was not shared by all NK-active clones established post-BMT. In fact, only six of 15 clones having a high degree of cytotoxicity for K562 were able to kill leukemic targets, again demonstrating the extensive heterogeneity of cells capable of mediating NK-like functions.

In part, our clonal analysis of cytotoxic cells post-BMT was facilitated by the method we used to prevent GVHD. More specifically, this patient received allogeneic marrow that was depleted of mature T cells by in vitro treatment with anti-T12 antibody and rabbit complement. He did not receive any other GVHD prophylaxis. T12 antigen is normally expressed on functionally mature T lymphocytes in peripheral blood and thymus but is not expressed on less-differentiated T cells within the thymus and, more importantly, is not expressed to any large extent on normal NK cells. The early proliferation of NK cells and delayed proliferation of mature T cells in our patient therefore likely reflects the in vitro treatment with anti-T12, which is more effective in depleting T cells than NK cells. NK cells normally reconstitute in the early posttransplant period and have been associated with acute GVHD, but NK cell proliferation would occur in the context of rapid T cell proliferation if T cells were not depleted from the donor marrow prior to transplantation. The finding that NK cells can reconstitute in large numbers without the development of clinically significant GVHD suggests that these cells are not responsible for GVHD. The additional finding that cloned NK cells from the donor are capable of cytotoxicity against the recipient's leukemic cells further suggests that it may be possible to distinguish graft-v-leukemia from GVHD following human marrow transplantation as has previously been demonstrated in murine experiments.

Although the present studies suggest that donor NK cells may play a role in preventing leukemia relapse after marrow transplantation, it should be emphasized that our studies were carried out in a single patient with T-ALL. We have not yet analyzed NK cells in other allogeneic transplant recipients with different types of leukemia or following autologous or syngeneic transplantation. The clonal analysis of cytotoxic effector cells is a laborious method that cannot easily be applied to large numbers of patients, but similar studies will have to be carried out in additional patients before the clinical significance of NK cells post-BMT is fully understood.

ACKNOWLEDGMENT

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