Generation of CD4+ Cytotoxic T-Lymphocyte Clones From a Patient With Severe Graft-Versus-Host Disease After Allogeneic Bone Marrow Transplantation: Implications for Graft-Versus-Leukemia Reactivity

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HLA-identical bone marrow transplantation (BMT) is associated with both graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) reactivity. Different T-cell subsets from the bone marrow (BM) graft may be responsible for GVHD and GVL reactivity after BMT. In the etiology of GVHD, not only CD8+ but also CD4+ donor T lymphocytes may play an important role. Here we report a patient with chronic myeloid leukemia (CML) who was transplanted with the BM from his HLA-genotypically identical sister. After BMT there was complete engraftment, but the patient died because of acute GVHD grade III-IV in complete remission. Cytotoxic T-lymphocyte (CTL) lines were generated after BMT using the irradiated leukemic cells from the patient as stimulator cells and the donor-originated peripheral blood mononuclear cells, procured from the patient after BMT, as responder cells. The generated CTL lines showed specific lysis of the recipient lymphocytes and leukemic cells in a 51Cr release assay. Two types of CTL clones could be established from these CTL lines, both phenotypically CD4+. Clone type I showed male-specific HLA-DQ5-restricted lysis of the recipient lymphocytes, but not of the circulating relatively mature leukemic cells from the patient. This may be explained by the low HLA-DQ5 expression of the more mature CML cells. Clone type II showed HLA-DR2-restricted minor histocompatibility antigen-specific lysis of the recipient lymphocytes and leukemic cells. Both types of CTL clones showed antigen-specific cell-mediated growth inhibition of the recipient clonogenic leukemic precursor cells. These CD4+ CTL clones produced several activating cytokines including tumor necrosis factor α, interferon γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage CSF. Our results illustrate that these CD4+ CTL clones may have induced GVHD directly by cytolyis and indirectly by activating cytokines. Because both types of CTL clones recognized the recipient leukemic progenitor cells, they may also contribute to GVL reactivity after BMT.

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MATERIALS AND METHODS

Generation of leukemia-reactive CTL clones. After informed consent, BM and peripheral blood (PB) were obtained from a 42-year-old patient with Philadelphia chromosome-positive (Ph+) CML transformed to the accelerated phase under treatment with hydroxyurea, and from his HLA-genotypically identical sibling BM donor.
Standard conditioning regimen for allogeneic BMT consisted of the combination of high-dose cyclophosphamide (60 mg/kg body weight, on days -6 and -5) and total body irradiation (900 cGy on day -1). To reduce GVHD, partial T-lymphocyte depletion from the BM graft was performed using counterflow centrifugation.\textsuperscript{27} Posttransplant immunosuppression consisted of cyclosporine A (3 mg/kg body weight/d) starting from day -1. After BMT there was complete engraftment, but the patient died on day 98 because of acute GVHD grade III-IV of the liver and intestinal tract in complete remission. HLA-typing was performed by standard serologic methods and by oligotyping. The HLA-typing of the recipient and his HLA-genotypically identical female donor was A1 A2 B7 B60(40) CW7 CW10 DR1 DR 15(2) DQ5 DQ6, DRB1*0101, DRB1*1501, DRB5*0101. Pretransplant primary mixed lymphocyte reaction (MLR) and cell-mediated (CML) assays between donor and recipient were negative.

To generate donor-antibody CTL lines, 2 x 10\(^6\) irradiated (30 Gy) leukemic PB mononuclear cells (PBMCs) (100% t(9;22) positive) from the patient were used as stimulator cells and 1 x 10\(^7\) donor- or PBMCs procured from the patient after BMT were used as responder cells. The cells were cultured at 37°C (5% CO\(_2\), 95% humidity) in RPMI medium plus 15% prescreened human AB serum in 50-mL tissue-culture flasks (Greiner, Alphen a/d Rijn, The Netherlands). The human AB serum was negative for hepatitis B surface antigen (HBsAg), human immunodeficiency virus, and alloantibodies against HLA antigens and was shown not to induce an autologous MLR. On days 9 and 14, T-cell growth factor (TCGF; Biotest, Offenbach, Germany) at a final concentration of 20% was added to the cells and the cells were restimulated with 10-fold of the irradiated leukemic cells from the recipient. On day 21 the established CTL line was further cloned by limiting dilution (effector cell concentrations of 3, 1, or 0.3 cells/well) in 96-well round-bottomed microtiter plates, 3 plates per concentration, in the presence of the irradiated (30 Gy) recipient leukemic cells (10\(^7\)/well) and in the presence of 20% TCGF and 300 IU/mL human recombinant IL-2. The generated CTL clones were further expanded in the presence of irradiated (30 Gy) EBV-LCL from the recipient and irradiated (30 Gy) allogeneic nonmatched PBMCs.

The phenotypes of the CTL clones were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) using CD3, CD4, and CD8 monoclonal antibodies (Becton Dickinson).

\textsuperscript{51}Cr release assay. Standard \textsuperscript{51}Cr release assays were performed as described.\textsuperscript{19} As effector cells, the generated CTL clones were used; as target cells, phytohemagglutinin (PHA)-stimulated lymphocytes, Epstein-Barr virus (EBV)-transformed B cells (EBV-LCL) or leukemic cells from the recipient, donor, and unrelated patients were used. \textsuperscript{51}Cr-labeled target cells were incubated with effector cells at various ratios. To measure spontaneous release of \textsuperscript{51}Cr, target suspensions were analyzed in the absence of effector cells. Maximum release was determined by adding 0.1 mL of the target suspension to 0.1 mL Zapoglobin solution (Coulter Electronics, Luton, UK).

The percentage specific lysis obtained in a \textsuperscript{51}Cr release assay was determined as follows: 100% x (Experimental Release cpm - Spontaneous Release cpm)/ (Maximum Release cpm - Spontaneous Release cpm).

Spontaneous release cpm/maximum release cpm ratio was less than 30% in the assays performed.

To determine CD8 and class I or CD4 and class II restriction of the recognition of the target cells, blocking studies were performed. Effector cells were incubated with saturating concentrations of anti-CD8 or anti-CD4 MoAbs or target cells with saturating concentrations of anti-class I (W6/32), anti-class II (PdVS), anti-DQ (SPVL3), anti-DQ5 (III8B1), anti-DQ6 (III8B3), or anti-DR (Becton Dickinson) MoAbs for 30 minutes before effector and target cells were cocultured.

To determine whether CML cells could competitively inhibit antigen-specific lysis by CTL clones, cold-target inhibition assays were performed. Non-\textsuperscript{51}Cr-labeled (cold) target cells were added to a specific combination of effector cells and \textsuperscript{51}Cr-labeled (hot) target cells (E:T ratio 10:1). The cold:hot target ratios used were 100:1, 30:1, 10:1, 3:1, and 1:1. The percentage inhibition of lysis of the hot target by cold targets was measured as follows: 100% x (% lysis of hot target only - % lysis of hot target in the presence of cold targets)\%/ lysis of hot target only.

PHA-stimulated lymphocytes were generated by stimulating 1 x 10\(^7\) PBMCs from the patient before BMT, the donor, or various unrelated donors with 0.2% PHA in RPMI plus 15% human AB serum for 3 days. The cells were then washed and further cultured in the absence of PHA and in the presence of IL-2 for at least 3 more days. Stable EBV-LCLs were established by in vitro transformation of 1 x 10\(^7\) PBMCs from the patient, donor, or various unrelated donors with EBV supernatant for 1.5 hours. The cells were washed and further expanded in RPMI plus 10% FCS (GIBCO Laboratories, Grand Island, NY). Both PHA-stimulated lymphocytes and EBV-LCL from the patient were generated from samples containing leukemic cells. However, cytogenetic analysis showed that these cells did not contain the t(9;22) translocation and, therefore, were not derived from the leukemic clone.

Leukemic cells were collected from the BM or PB after informed consent of the recipient and of unrelated patients with acute myeloid leukemia (AML) (>95% morphologically recognizable leukemic cells) or CML at diagnosis. The cells were centrifuged over Ficoll Isopaque (density, 1.077 g/cm\(^3\), 1,000g, 20 minutes) and the interphase cells were procured and cryopreserved in liquid nitrogen. Before use, the cells were thawed, washed twice, and resuspended in RPMI containing 15% prescreened AB serum.

Cell-mediated inhibition of clonalogenic (leukemic) precursor cell growth. To determine specific reactivity of the generated CTL clones with the clonalogenic leukemic precursor cells from the patient and with the hematopoietic progenitor cells from the HLA-identical donor, 2 x 10\(^7\) target cells (leukemic cells from the recipient or low-density BM cells from the donor) in 0.1 mL medium consisting of Iscove’s modified Dulbecco’s medium (IMDM) plus 15% human AB serum was mixed with the generated irradiated (20 Gy) CTLs at various effector/target (E:T) ratios. The cell mixture was centrifuged (1,000g, 15s) to establish cell-cell contact between CTL and the target cells, and then precultivated for 4 hours at 37°C. After inoculation, the cells were resuspended and cultured as a single cell suspension at a concentration of 2 x 10\(^6\) target cells/mL in a 30-mm culture dish in 1 mL IMDM containing 10% prescreened human AB serum, 2 U/mL erythropoietin (Clag AG International, Zug, Switzerland), 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Sandoz Ltd, Basel, Switzerland), 500 U/mL macrophage CSF (Cetus Corp, Emeryville, CA), 50 ng/mL SCF (Amgen, Thousand Oaks, CA), 25 mg/mL IL-3 (Sandoz Ltd, Basel, Switzerland), 0.47 g/L human transferrin (Behringwerke AG, Marburg, Germany) saturated with FeCl\(_3\) 5 x 10\(^{-4}\) mol/L mercaptoethanol, and mideleylulose (Methocel 4,000 cps, Fluka, Freiburg, Germany) at a final concentration of 1.1% (wt/vol). Because an increased growth of colony-forming unit-monoocyte (CFU-M) colonies could be observed when the generated CTL clones were used as effector cells compared with the untreated control cultures, a conditioned medium was made from these CTL clones. The conditioned medium was added to all culture dishes at a final concentration of 20% to obtain maximum humoral stimulation for colony growth. The cells were incubated in a fully humidified atmosphere of 5% CO\(_2\) and 37°C. After 14 days of culture, the number of CFU-granulocyte colonies (defined as ag-
aggregates of more than 50 neutrophilic or eosinophilic granulocytes), CFU-M (defined as aggregates of more than 50 monocyctic cells), and burst-forming unit-erythroid colonies (defined as colonies consisting of more than 50 hemoglobinized cells) were scored, using an inverted microscope. The colony growth in the presence of effector cells is expressed as percentage of growth of the number of colonies observed in the untreated control culture (grown in the absence of effector cells). To control for antigen-nonspecific inhibition of colony growth caused by secretion of humoral inhibitory factors into the culture medium, effectors were also plated together with the target cells at the same E/T ratios directly in the semisolid medium, without 4-hour preincubation, to prevent cell-cell contact. 29

Cytokine gene expression in CTL clones. To eliminate residual contaminating feeder cells from the generated CTL clones, the clones were purified using fluorescence-activated cell sorting using CD4 antibodies. The clones were analyzed during steady-state phase of proliferation 4 days after addition of feeder cells consisting of EBV-LCLs and third-party mononuclear cells. Cytokine gene expression in these selected cells was analyzed by cDNA synthesis and polymerase chain reaction (PCR) amplification as previously described. 20,21 From 2 µg of total cellular RNA samples, the mRNA fraction was reverse transcribed into cDNA. Oligonucleotide primer sequences were chosen from separate exons of the studied genes to differentiate cDNA amplification products from contaminating genomic DNA products. Thirty-three PCR cycles were performed. Samples of each PCR reaction mixture were electrophoresed in 2% agarose gels. Gels were stained with ethidium bromide and photographed.

RESULTS

A donor-derived CTL line was generated that showed specific lysis of the recipient lymphocytes (54%) and low specific lysis of the recipient leukemic cells (13%) in a 31Cr release assay. The donor cells (autologous control) were not recognized. After cloning by limiting dilution, four stable CTL clones were established that showed specific lysis of the recipient lymphocytes. When these four clones were further expanded, two types of clones could be recognized based on their specific reactivity in the 31Cr release assay (Fig 1). No CD8+ CTL clones were established. Clone type I (n = 2), phenotypically CD4+, showed specific lysis of the recipient lymphocytes and EBV-LCLs, but not of the leukemic cells from the recipient. Clone type II (n = 2), also CD4+, showed specific reactivity with the recipient lymphocytes, EBV-LCLs and leukemic cells. EBV-LCL from the donors were not recognized by both types of clones.

To determine class II restriction of the recognition of the target cells blocking studies were performed. The specific lysis of the recipient EBV-LCL by clone type I could be blocked with anti-class II (DR‘DQ’DP), anti-DQ and anti-DQ5 MoAbs illustrating DQ5-restricted recognition (Fig 2). The specific lysis of the recipient lymphocytes and leukemic cells could also be blocked by these MoAbs (data not shown). The specific lysis by both types of CTL clones could further be blocked with anti-CD4 MoAbs (data not shown).

To analyze the specificity of clone type I and II, the CTL clones were tested in a 31Cr release against a family sharing the HLA-class II antigens with donor and recipient (Table 1). Clone type I only recognized targets from male individuals expressing the HLA-DQ5 antigen. Sibling 4, a female, was not lysed by clone type I. When clone type I was further tested against EBV-LCLs from unrelated donors, only male (n = 7) but not female donors (n = 6) were recognized by clone type I, illustrating male specificity (Fig 3). Clone type II only recognized the targets sharing the HLA-DR2 antigen with recipient and donor, but not all of them. The HLA-DR2 molecule was present only in haplotypes a and c. Sibling 4, sharing the HLA-DR2 haplotype c with the mother was not recognized, whereas the mother was recognized, illustrating mH antigen recognition by clone type II. Cross-over could be excluded by oligotyping. When clone type II was further tested against EBV-LCLs from an unrelated HLA genotypically identical sibling pair sharing the HLA-DR2 antigen with the donor, only one of the two siblings was recognized (specific lysis responses,
was directed against a mH antigen, and that the recognition of the recipient lymphocytes and EBV-LCLs, and clone type II, the CD4+ CTL clone that recognized the recipient lymphocytes, leukemic cells, and EBV-LCLs. The specific lysis by clone type I of the recipient EBV-LCL could be blocked with anti-class II (DR’DQ’DP) MoAbs. The specific lysis by clone type II could be blocked with anti-DR and anti-class II (DR’DQ’DP) MoAbs. Lysis of the target cells was measured using a standard 51Cr release assay in duplicate at E/T ratio 2:1. Numbers indicate means ± SD (n = 3).

30% and −8%). These results illustrated that clone type II was directed against a mH antigen, and that the recognition was HLA-DR2 restricted.

Although clone types I and II were generated against the irradiated recipient leukemic cells, CTL clone type I only recognized the recipient lymphocytes and EBV-LCL and not the recipient leukemic cells in the 51Cr release assay. This CTL clone was further tested against CML cells from two other HLA-DRQ5+ male patients, leukemic cells from HLA-DQ5+ male patients with AML, acute lymphoblastic leukemia (ALL), or normal BM, and against DOHH2, an immunoblastic lymphoma cell line generated from an HLA-DR1,DQ5,DR2+ male patient. Only the CML samples were not recognized by clone type I (Fig 4). When CML samples were used as competitors in a cold target inhibition assay, the specific lysis by CTL clone type I of the EBV-LCLs from the patient could not be inhibited by the CML cells from the patient or another HLA-DQ5+ CML target from a male patient (Fig 5). The EBV-LCL from the patient (autologous control) and the HLA-DQ5+ AML-M4 target from a male patient could strongly inhibit the specific lysis of EBV-LCL. Fluorescence-activated cell sorting (FACS) analysis of the tested cells showed that the HLA-DQ expression by CML cells as measured by the mean fluorescence peak was 10-fold lower than the expression of HLA-DR molecules (data not shown), explaining the inability of this type I clone to recognize male CML cells. The expression of HLA-DR and DQ-antigens was similar on AML and ALL cells.

When CTL clone type II was tested against AML cells from HLA-DR2+ unrelated patients, two of three samples were lysed (specific lysis >50%), illustrating mH antigen-specific lysis of not only CMLs, but also AML targets.

To determine specific reactivity of clone type I and II with the recipient clonogenic leukemic precursor cells, the recipient leukemic cells were used as target cells in a clonogenic cell-mediated cytotoxicity assay as described in Materials and Methods. Control cultures showed that an increased growth of CFU-M colonies was observed in the presence of conditioned media from the CD4+ CTL clones compared with the control culture, indicating secretion of a humoral stimulatory factor into the culture medium by these CTL clones. Both CTL clone type I and type II showed mH antigen-specific growth inhibition of the leukemic precursor cells from the recipient (Fig 6). The hematopoietic progenitor cells from the HLA-genotypically identical donor (autologous control) were not recognized by both types of CTL clones.

Cytokine gene expression in the generated CTL clones. Both types of CTL clones showed expression of IL-2, tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and GM-CSF genes and no expression of IL-1α, IL-1β, IL-4, IL-6, and IL-12 genes as measured by PCR analysis. CTL clone

![Table 1. Differential Recognition of an Unrelated HLA-DR1DQ5DR2+ Family by Clone Types I and II](image)
type II but not type I also showed expression of M-CSF and IL-10 genes.

DISCUSSION

Several studies have indicated that both CD8⁺ cytotoxic T lymphocytes and IL-2–secreting CD4⁺ and/or CD8⁺ T cells may play a role in the etiology of GVHD. In this study, we show the generation of CD4⁺ CTL clones from a male patient with GVHD grade III-IV, who was transplanted for CML with the HLA-genotypically identical BM from his sister. Two types of CTL clones could be established. Clone type I showed DQ5-restricted male-specific lysis of the recipient lymphocytes and EBV-LCLs, but not the leukemic cells, whereas clone type II showed DR-2-restricted mH antigen-specific lysis of the recipient lymphocytes, EBV-LCL and leukemic cells. Although this CTL clone type I was generated against the recipient leukemic cells, the recipient CML cells and CML cells from two other male HLA-DQ5⁺ patients were not recognized in a ⁵¹Cr release assay. Cold target inhibition experiments confirmed that the HLA-DQ5-restricted male-specific antigen is not adequately presented on the majority of CML cells. FACS analysis of these leukemic cells showed low expression of the HLA-DQ5 restriction molecule, explaining the inability of the CTL clone to recognize these cells. IFN-γ, which may upregulate class II expression, did not influence the recognition of the CML cells by these clones. AML and ALL cells from two other male HLA-DQ5⁺ patients, both expressing the HLA-DQ5 molecule as measured by FACS analysis, were recognized by CTL clone type I. HLA class I-restricted recognition of male-specific antigens (H-Y antigen) by CD8⁺ CTLs has been shown previously. It is not

![Fig 3. Specificity of clone type I, that showed HLA-DQ5-restricted lysis of the recipient lymphocytes and EBV-LCLs. Lysis of DOB⁺ EBV-LCLs from unrelated donors was measured using a standard ⁵¹Cr release assay in duplicate at E/T ratio 5:1 (n = 2). Only the male donors were recognized by the CTL clone, illustrating male specificity.](image)

![Fig 4. Specific recognition of leukemic cells by clone type I, that showed male-specific HLA-DQ5-restricted recognition of the recipient lymphocytes and EBV-LCLs, but not of the recipient leukemic cells. Clone type I also did not recognize CML cells from two other HLA-DQ5⁺ male patients, but lysed the HLA-DQ5⁺ immunoblastic lymphoma cell line, DOHH2, and cells from three DQ5⁺ male patients with AML (M4), C-ALL, or normal BM, respectively, using a standard ⁵¹Cr release assay in duplicate at E/T ratio 20:1. Numbers indicate means ± SD (n = 6).](image)
known whether the mH antigen recognized in the context of the HLA class II molecule is similar to the H-Y antigen recognized in the context of HLA class I molecules, because the peptide recognized in association with the HLA molecules have not yet been identified. In general, CD8+ T lymphocytes recognize endogenously processed peptides in the context of HLA class II molecules, whereas CD4+ T lymphocytes recognize peptides derived from extracellular sources in the context of HLA class I molecules. However, presentation of endogenous peptides by HLA-DR molecules and the recognition of these peptides by CD4+ CTLs have been described. Here we show the recognition of an endogenous male-specific antigen in the context of HLA-DQ5 by CD4+ CTLs. Thus, both CD4+ and CD8+ male-specific clones are capable of lysis of clonogenic leukemic precursor cells (Fig 6). Therefore, these CTLs may contribute to the GVL effect after BMT in case of a female donor and a male patient. Clone type II, also generated against the recipient leukemic cells, showed specific reactivity with the recipient leukemic cells as well as with the recipient leukemic precursor cells, indicating that these CTL clones may have exhibited antileukemic reactivity after BMT. Clone type II also showed reactivity with AML samples from two HLA-DR2+ patients. Furthermore, these clones also showed HLA-DR2-restricted specific growth inhibition of leukemic precursor cells from another CML patient but also from normal individuals (data not shown). These results illustrate that the antigen recognized by CTL clone type II is not the peptide fusion product of the t(9;22) chromosomal translocation, characteristic for CML. Recently, Oettel et al described the generation of CTL lines and clones against t(9;22)-positive EBV-LCLs that showed reduced reactivity against t(9;22)-negative EBV-LCLs from the same patient. However, after prolonged culture of these CTLs, both t(9;22)-positive and -negative targets were recognized. Thus, both studies illustrate that in these recognitions, the product of the t(9;22) fusion gene was not the target structure of these alloreactive T cells.

Besides a cytolytic function a stimulatory effect was also observed when the CTL clones were tested in the clonogenic assay. PCR analysis of both types of CTL clones showed expression of IL-2, TNF-α, IFN-γ, and GM-CSF. Recent experimental data emphasized the role of various cytokines including TNF-α, IFN-γ, IL-1, IL-2, IL-3, GM-CSF, and M-CSF in the induction of GVHD. CTL clone type I expressed the classical Th1 phenotype of cytokines, a cytokine pattern that is also produced by CD8+ CTLs. The cytokine genes expression by CTL clone type II did not discriminate to distinct patterns of the Th1 or Th2 types. Besides the Th1 phenotype of cytokines, CTL clone type II expressed IL-10 and M-CSF genes. Elevated levels of M-CSF in the serum of mice suffering from GVHD have been described. The mechanism by which M-CSF may contribute to the induction of GVHD is not clear. By activation of the antigen-p呈示ing cells, these cells in turn may restimulate the primed T cells. Furthermore, activated macrophages can release TNF-α, an inflammatory cytokine that has been shown in mice to be a central mediator of GVHD, resulting in cytolsis, directly or indirectly.

In conclusion, we have shown that alloreactive, malespecific and leukemia-reactive CD4+ CTL clones were generated from a patient with severe GVHD after HLA-genotypically identical BMT for CML. These CTL clones may have induced GVHD directly by cytolsis and indirectly by the
secretion of activating cytokines and may have contributed to GVL reactivity after BMT.

ACKNOWLEDGMENT

The authors thank Dr A. Mulder for supplying the MoAbs and J. Parlevliet for performing the HLA- and oligo-typing.

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