A Method for the Production of CD4+ Chronic Myelogenous Leukemia-specific Allogeneic T Lymphocytes

Jonathan S. Serody, Mark E. Brecher, Georgette Dent, Stuart A. Bentley, Jeffrey A. Frelinger, and Thomas C. Shea

ABSTRACT

The graft-versus-leukemia effect is critical to the maintenance of remission in patients transplanted for the treatment of chronic myelogenous leukemia (CML). A pivotal issue in transplantation for CML is whether donor lymphocytes are specific for host tumor or myeloid cells or a subset of the lymphocytes that cause graft-versus-host disease. We have enrolled seven patients in an experimental trial to evaluate the specificity of HLA-matched donor lymphocytes in vitro. We have produced 11 CD4+ cytotoxic and proliferative T-cell clones from five of the donors that only lyse or proliferate to leukemic myeloid cells. These T lymphocytes do not react with interleukin (IL)-2-stimulated blasts, natural killer-sensitive targets, donor neutrophils, or bcr-abl+ EBV-lymphoblastoid cell lines. We show that the addition of the cytokines IL-7 and IL-12 during the production of T-cell clones enhances the recovery of myeloid-specific clones in vitro. Five of the myeloid-specific clones that we produced maintained specificity over 12 weeks in culture. Adoption of this method should allow for the expansion and in vitro testing of CML-specific CD4+ T-cell clones in adoptive immunotherapy.

INTRODUCTION

Allogeneic BMT is the only proven curative therapy for the treatment of CML (1, 2). The success of allogeneic BMT is due to two critical factors. (a) The conditioning regimen destroys malignant cells in the bone marrow and provides a mechanism for engraftment of donor stem cells. (b) Donor T lymphocytes have been shown to be pivotal in the prevention of relapse after BMT for CML (3).

The initial studies that showed a benefit in preventing relapse by the inclusion of donor T lymphocytes were in the setting of HLA-matched T-cell-depleted sibling BMT. Several groups (3-5) demonstrated a 6-8-fold increase in the risk of relapse in patients who received T-cell-depleted marrow compared with unmanipulated marrow. This increased risk of relapse offsets the decreased risk of acute and chronic GVHD and led to an overall decreased survival in these patients.

The profound effect of donor T lymphocytes in eliminating CML myeloid cells has been shown convincingly by several groups (6-10). These studies have demonstrated that the infusion of donor mononuclear cells resulted in complete remission as demonstrated by histology, cytogenetic evaluation, and molecular methods. The side effects of infusion of donor buffy coat mononuclear cells are most commonly acute and chronic GVHD and pancytopenia. Recently, Mackinnon et al. (11) showed that the infusion of a limited number of CD3+ T lymphocytes could induce remissions with a minimal incidence of GVHD.

Previous in vitro work showed that NK cells and CD4+ MHC-restricted and CD8+ MHC-restricted lymphocytes are all implicated in the GvL response (12-14). Since December 1994, we have enrolled seven patients with CML who were preparing to undergo an HLA-identical sibling BMT on an experimental trial to evaluate whether myeloid-specific T lymphocytes could be produced from the donor in vitro. We have produced 11 CD4+ T-cell clones that show enhanced activity against CML cells. Five of these T-cell clones have maintained tumor specificity over 12 weeks while maintained in culture. None of these CML-specific clones proliferated or lysed when stimulated by EBV-LCLs from either the donors or recipients, HLA-mismatched allogeneic CML cells, IL-2-stimulated T lymphoblasts, K562 cells, or HL-60 cells. This method may be applicable to large-scale production of tumor-specific lymphocytes for adoptive immunotherapy.

MATERIALS AND METHODS

The HLA type of the seven patients who entered the study is given in Table 1. Only DR typing was available on one donor/patient pair UPN 35. HLA typing was performed by serology at the University of North Carolina Tissue Typing Laboratory. Class II DNA typing was performed using single-strand oligonucleotide primers at Roche Laboratory (Burlington, NC). All patients and donors who entered into this study signed informed consent. The protocol was approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina School of Medicine.

Patients with a diagnosis of CML who had a HLA-identical donor were eligible for participation in this trial. CML was diagnosed by the presence of the 9;22 translocation in samples of bone marrow, in conjunction with histology from the bone marrow aspirate and biopsy. Granulocyte-enriched fractions from peripheral blood (termed myeloid tumor cells in the text) were collected from patients with CML using a COBE spectra (Lakewood, CO). Seven to eight liters of whole blood were processed at flow rates of 50-70 ml/min. Five hundred ml of hydroxyethyl starch (HES) were used as an erythrocyte-sedimenting agent, and the citrate to whole-blood ratio was 13:1. We collected approximately 10^12-10^13 myeloid cells per patient.

The collected cells were mixed in a 1:1 ratio with 60% plasma, 20% human serum, and 20% DMSO. The final concentration of cells was adjusted to less than 5 x 10^6 cells/ml. The leukapheresis product was frozen using a rate-controlled freezer (Cryomed, Marietta, OH) and stored in liquid nitrogen at -196°C. Mononuclear cells from patients were collected separately during leukapheresis and frozen as described above. Mononuclear cells were isolated from the donor by a standard Ficoll-Hypaque separation. EBV-LCLs were produced as described previously (15). K562 and HL-60 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% (v/v) FCS, 2 mm L-glutamine, and 5 x 10^-5 M 2-mercaptoethanol (R10) and Iscove’s DMEM with similar supplements.

Generation of T-Cell Lines. T-cell lines were produced using a modification of an established technique (13). Peripheral blood mononuclear cells (10^7) from the donor were incubated with 5 x 10^9 irradiated (3000 Gy; Atomic Energy of Canada, Ottawa, CA) myeloid tumor cells from the recipient in 24-well plates (Costar, Cambridge, MA) in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 15% (v/v) AB+ human serum (Sigma Chemicals, St. Louis, MO), 10% TCGF (Biotest, Dreieich, Germany), 2 mm L-glutamine, and 2-mercaptoethanol (R15). Six days later fresh media was added. The mononuclear cells were restimulated with a 10-fold excess of CML cells at day 13.

PCR. We used PCR to specifically amplify the bcr-abl region from EBV-LCLs produced by patients with CML. Briefly, mRNA was isolated from the leukocytes.
Table 1 Characteristics of donor/patient pairs

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<th>UPN no. 35</th>
<th>UPN no. 65</th>
<th>UPN no. 98</th>
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<td>F/F</td>
<td>M/M and F</td>
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<td>10 mo</td>
<td>6 mo</td>
<td>5 mo</td>
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<td>CML-specific clones</td>
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<td>RB D4, F9, B3, E2, H6</td>
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<td>B7, 51, Cw2</td>
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<td>DR4, 13, DRw52</td>
<td>DR10, 13, DRw52</td>
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* UPN, unique patient number; F, female; CP, chronic phase.

Table 2

<table>
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<tr>
<th>CML Cells</th>
<th>EBV-LCL donor</th>
<th>EBV-LCL host</th>
<th>K562</th>
<th>CML Cells mismatched</th>
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| Fig. 1. Lytic activity of representative clones. Clones from lines RB, LS, and SM were screened for lytic activity in a conventional 51Cr release assay. Six clones demonstrated lytic activity against only CML cells from recipients with CML. The E:T ratio for maximal lysis was 10:1. The spontaneous release from the assay for all target cells, including CML cells and neutrophils, was <20%. Assays were performed in either duplicate or triplicate. Target cells are indicated in the legend at the right. This is one of three representative examples for each of the clones tested. Mismatched CML cells are mismatched at all class II MHC loci. Positive clones were identified as clones that had a specific lysis of two times any other target cell for all three assays performed. All samples were run in triplicate. IL-2-stimulated lymphocytes were produced using a previously described procedure (15). |
PRODUCTION OF CD4+ CML-SPECIFIC T CELLS

T-Cell Cloning. T-cell lines were cloned in 96-well plates (Costar, Cambridge, MA) at 0.3 cells/well in the presence of 5 × 10⁴ CML cells, 20% TCGF, 20 mg/ml IL-7 (Endogen, Cambridge, MA), and 10 IU/ml IL-12 (R&D Systems, Inc., Minneapolis, MN). The fraction of growth-positive wells in cloning plates was stimulated in a similar manner weekly. After approximately 2 weeks, T-cell clones were transferred to 24-well plates and assayed for activity as described. EBV-LCLs from the patient were included as feeder cells when clones were isolated from lines RB, GH, HH, LS, and SM. For the last three donor/patient pairs, duplicate T-cell cloning plates were set up with cells incubated in the presence of either TCGF only or TCGF plus IL-7 and IL-12. All other clones were prepared in media containing IL-7 and IL-12.

Flow Cytometry. The surface phenotype of T-cell clones was analyzed by flow cytometry using a FACSScan (Becton Dickinson, Mountain View, CA). FITC-conjugated CD4 (mouse IgG1), FITC-conjugated CD8 (mouse IgG1), and PE-conjugated CD3ε (mouse IgG1) were used (PharMingen, San Diego, CA) for two-color flow cytometry. T-cell clones were incubated for 30 min at 4°C with 1 µg of FITC-conjugated and PE-conjugated antibody per 10⁶ T cells. Gating was performed using forward and side-scatter parameters. Ten thousand events were collected, and histograms were analyzed using Cicero software (Cytomation, Fort Collins, CO).

The surface phenotype of the collected myeloid cells was analyzed by two-color flow cytometry. FITC-conjugated CD19 (mouse IgG1; PharMingen), FITC-conjugated CD1a (mouse IgG1; PharMingen), FITC-conjugated CD16 (mouse IgG1; PharMingen), and PE-conjugated CD3ε were used. Twenty thousand events were collected and analyzed.

Statistics. Groups were compared for significant differences using χ² analysis with Yates correction for continuity.

RESULTS

Activity of Bulk Lymphocyte Lines. Seven patients entered this experimental trial. The characteristics of the seven donor/patient pairs are found in Table 1. One of the donors had two HLA-matched sibling donors available. Therefore, we produced clones from eight donors using CML cells from seven recipients. We were unable to produce myeloid-specific T lymphocytes from three of the donor/patient pairs. T-cell lines from the four donor/patient groups were produced as described in the text. All of these lines showed activity against multiple target cells, including CML cells (data not shown). There was no proliferative or lytic activity demonstrated from three bulk cultures, which corresponded to the inability to produce myeloid-specific clones from these lines.

Using two-color flow cytometry, we analyzed the population of cells included in the granulocyte-enriched fraction of myeloid cells from recipients diagnosed with CML. Myeloid cells (10⁶) from three different patients were analyzed for expression of CD16 (neutrophil marker), CD3ε (T-lymphocyte marker), CD1a (dendritic cell marker), and CD19 (B-cell marker) using two-color flow cytometry. Greater than 99% of the myeloid cells expressed only CD16 using two-color flow cytometry (data not shown). None of the cells expressed CD1a, and <1% expressed either CD19 or CD3ε. These data demonstrate
that at least 99% of the target cell population in the granulocyte fraction were myeloid cells (referred to as tumor cells in the remainder of the text, although not all of the cells are tumor cells).

Specificity of T-Cell Clones. T cells were cloned from bulk lymphocyte lines as described. The first 50 consecutive clones from cloning plates that expanded within 30 days to a total number of 10^5 clones were tested for the ability to specifically lyse or proliferate to tumor cells for each donor/patient pair. All of the clones were from plates in which <37% of the wells were growth positive and therefore assumed to be clones. All 358 clones were screened for cytolytic activity in 51Cr release assays. Clones that did not demonstrate lytic activity against any of the target cells tested were screened for proliferation to specific target cells. Six T-cell clones that specifically lysed tumor cells were isolated (see Fig. 1). Three clones (LS E9, LS D3, and SM E2) showed markedly enhanced lytic activity against HLA-matched tumor cells. The other three clones showed a moderate level of specific lytic activity toward tumor cells. These clones did not lyse EBV-LCLs from the donor or recipient, the NK-sensitive cell line K562, IL-2-stimulated T lymphocytes, or HLA-mismatched tumor cells.

Seven clones from three donors specifically proliferated when HLA-matched tumor cells were used as stimulator cells. These clones did not proliferate when EBV-LCLs from the donor or patient, IL-2-stimulated lymphocytes from the patient, or HLA-mismatched myeloid cells were used as stimulator cells. Using PCR, we demonstrated the presence of the bcr-abl mRNA in EBV-LCLs from these three patients (data not shown). As shown in Fig. 2, the seven T-cell clones that specifically proliferated when incubated with myeloid cells from the patients did not proliferate when incubated with the bcr-abl+ EBV-LCLs from these patients, suggesting that peptides from the bcr-abl fusion protein are not the antigen that is recognized. One clone, HHB2, proliferated to both myeloid cells and EBV-LCLs of the recipient. This clone, which is CD4+, is currently being evaluated for bcr-abl-specific activity.

We investigated the long-term specificity of five of the CML-specific clones LS E9, LS D3, SM E2, GH E10, and GH H3. These clones were maintained in culture and stimulated weekly with HLA-matched CML cells and TCGF. After 12 weeks in culture, the clones were tested for their ability to proliferate against or lyse tumor target cells. The five clones maintained their specificity during this time (two of the clones are shown in Fig. 3). Of interest, the clones did not specifically proliferate to or lyse normal neutrophils from the HLA-matched sibling donors.

The total activity of the 358 clones that were tested is shown in Table 2. Two hundred seventy-nine of the clones did not lyse or proliferate to any of the target cells tested. Twenty-seven of the clones lysed or proliferated when incubated with EBV-LCLs from the donor and recipient. Flow cytometry revealed 15 of 15 of the lytic clones to be CD3+/8+/4—, the typical phenotype of EBV-specific CTLs. Forty-one of the clones either lysed or proliferated to multiple target cells, including tumor cells, IL-2-stimulated lymphocytes, and EBV-LCLs from both the donor and the recipient. Thus, the majority of clones that were isolated by this method did not have effector activity against any of the target cells in either 51Cr release or [3H]thymidine proliferation assays.

From the last three donor/patient pairs, we analyzed the contribution of the cytokines IL-7 and IL-12 in the production of myeloid-specific clones. We were unable to isolate tumor-specific clones in the presence of TCGF alone. However, from duplicate cloning plates prepared at the same time in the presence of IL-7 and IL-12, we isolated five tumor-specific clones from the 144 clones tested (P = 0.07). Thus, there was a statistical trend for IL-7 and IL-12 to increase the ability to recover tumor-specific T-cell clones.

Flow Cytometry. We performed two-color flow cytometry on the tumor-specific T-cell clones and on 15 lytic EBV-specific T-cell clones. All of these EBV-specific T-cell clones expressed CD3 and CD8 (see Fig. 4). This demonstrates that CD8+ T lymphocytes can be produced using our in vitro system when EBV-LCLs are used as feeder cells. All 11 of the tumor-specific T-cell clones expressed CD3 and CD4 (see Fig. 4). None of the tumor-specific T-cell clones expressed CD8.

DISCUSSION

The GvL response is a complex phenomenon that involves multiple effector populations (12–14). Previous investigators have shown that
Fig. 4. Flow cytometry profile of selected clones. Two-color flow cytometry was performed as indicated in the text. Three representative clones are demonstrated. A, top two histograms are the flow cytometry results on clone GH H3, a CML-specific proliferative clone. This clone shows the surface phenotype CD3+/4-/8-. B, the second set of histograms characterize a second CML-specific proliferative clone GH E10. This clone also expresses CD3+/4-/8-. C, CML-specific lyric clone RB C6. This clone expresses a CD3+/4+ phenotype. D, clone GH G11, which is a CD3+/8+ EBV-specific clone, is also demonstrated. All of the remaining CML-specific clones expressed the CD3+/4-/8- phenotype.

both minor antigen and alloreactive T lymphocytes can lyse CML myeloid cells (13, 18). However, previous investigators have not been able to produce T cells with enhanced activity against only myeloid tumor cells. We describe a method for the production of CD4+ tumor-specific T lymphocytes. This process involves the expansion of HLA-matched sibling allogeneic T cells in the presence of patient tumor cells, donor EBV-LCLs, and the cytokines IL-2, IL-7, and IL-12.

We found several different patterns of activity from our T-cell clones. Seven of the clones specifically proliferated when incubated with tumor cells from the recipients and had no response when stimulated by other cells. The majority of T cells (279 clones) that were isolated did not lyse or proliferate to any of the target cells. From seven donor/recipient pairs, 27 T-cell clones were isolated that were specific for EBV proteins because the clones lysed or proliferated when incubated with target cells from both the recipient and donor that were transformed with EBV. Fifteen of fifteen of these lyric clones expressed CD3+/8+/4-. Despite the presence of NK activity, as shown by lysis of K562 cells from bulk lines, we were not able to isolate clones with NK activity.
Our data concerning the maintenance of specificity of our T-cell clones differs from that of Oettel et al. (18), who investigated the specificity of allogeneic T cells in a CML model. They previously published that bcr-abl-specific allogeneic T cells could be produced, but that these T cells lost their specificity in culture and began to kill bcr-abl-negative EBV-LCLs. They concluded that the recognition of bcr-abl by these T cells was a part of the allosresponse and was not a separate phenomenon. We have shown that we can maintain CML-specific T-cell clones for 12 weeks in culture with little change in the specificity of these clones. These clones do not recognize bcr-abl+ target cells or host neutrophils. These data suggest recognition by these clones of early myeloid proteins or proteins preferentially produced in myeloid cells but not from the bcr-abl fusion protein.

Our system differs from that of Oettel et al. (18) in two fundamental ways. (a) We used the granulocyte fraction from patients with CML to stimulate mononuclear cells. This fraction contains ~99% myeloblasts to mature neutrophils. Oettel et al. (18) used bcr-abl+ EBV-LCLs to stimulate their lymphocytes. (b) We used mononuclear cells from HLA-matched siblings to produce our T-cell clones. The previous group used HLA-disparate mononuclear cells to produce T-cell clones. Naturally, this system is dominated by the extremely strong allosresponse.

Recently, Faber et al. (19) used a similar method to produce CML-specific T lymphocytes. They generated CD4+ T-cell clones with poor lytic activity when irradiated CML cells were incubated with HLA-identical mononuclear cells. The addition of IFN-α to the culture resulted in the growth of CD8+ lymphocytes with enhanced lytic activity. However, they were not able to produce T lymphocytes that lysed only host myeloid tumor cells. These results are somewhat different from ours. We believe that these differences may be explained by the different methods and culture systems that we used. Faber et al. (19) screened only 26 CD4+ clones and did not evaluate the proliferative activity of these clones. They did not use cytokines such as IL-7 or IL-12 (which we have shown increase the recovery of tumor-specific T-cell clones), whereas we did not include IFN-α. Furthermore, as we have shown, in three donor patient pairs we were not able to produce tumor-specific T-cell clones.

Previous investigators have evaluated the T-lymphocyte response to the bcr-abl fusion protein characteristic of CML. These models consistently demonstrate that class II-restricted lymphocytes are produced when bcr-abl fusion peptides are used to stimulate mononuclear cells (20). ten Bosch et al. (21) studied the in vitro response to bcr-abl peptides using human T lymphocytes. They found that the lymphocytes that recognized this peptide were restricted by the class II molecule DR2. The production of myeloid-specific class I-restricted lymphocytes has not been seen in these model systems.

Bocchia et al. (22) previously tested the ability of class I alleles to bind peptides from the fusion region of the bcr-abl protein. They found several peptides from bcr-abl that bound to the HLA molecules A3, A11, and B8. Although none of our patients expressed HLA A11 or HLA B8, four of them expressed the HLA molecule A3. We were not able to isolate CD8+ myeloid-specific lymphocytes from these patients despite the ability of bcr-abl peptides to bind to HLA A3 with moderate affinity. To ascertain whether our data represent an artifact or HLA-matched siblings to produce our T-cell clones. The previous group used HLA-disparate mononuclear cells to produce T-cell clones. Naturally, this system is dominated by the extremely strong allosresponse.

Our demonstration that all of the tumor-specific lymphocytes expressed CD4 was unexpected. We evaluated a large number of clones to identify class I-restricted CML-specific T cells. Despite screening 358 clones, we did not identify CD8+ tumor-specific T-cell clones. Our system for generating T-cell clones was adapted from that used in our laboratory to successfully isolate CD8+ HIV-specific T-cell clones (17). We were able to show that EBV-restricted CD8+ CTLs can be produced using EBV-LCLs that are bcr-abl+. This would make it less likely that the absence of CD8+ tumor-specific T cells is an artifact of the in vitro system.

In conclusion, we have established a method for the production of CD4+ T lymphocytes with enhanced proliferative and lytic activity toward HLA-matched myeloid cells from recipients with CML. Our data show that the cytokines IL-7 and IL-12 enhance the recovery of tumor-specific T lymphocytes. We have shown that we can maintain the specificity of these tumor-specific T-cell clones in vitro for up to 12 weeks. This method, however, was only successful in expanding tumor-specific T-cell clones in four of the seven donor patient pairs. If these cells can be sufficiently expanded in vitro, the infusion of tumor-specific T-cell clones should provide evidence that the effector cells that mediate GvL can be separate from those that mediate GVHD.

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