Graft Failure After T-Cell–Depleted Human Leukocyte Antigen Identical Marrow Transplants for Leukemia: II. In Vitro Analyses of Host Effector Mechanisms

By Claudio Bordignon, Carolyn A. Keever, Trudy N. Small, Neal Flomenberg, Bo Dupont, Richard J. O'Reilly, and Nancy A. Kernan

To identify mechanisms potentially contributing to graft failure, 19 leukemic recipients of T-cell–depleted marrow transplants who failed to engraft following a transplant of HLA identical sibling marrow depleted of T cells by soybean agglutinin (SBA) and sheep erythrocytes (E) were evaluated. Peripheral blood mononuclear cells isolated at the time of failure were consistently of host origin, bearing the phenotype of suppressor T cells (CD3+, CD8+, Leu 7+). A direct cytolytic effect on 51Cr-labeled donor-derived target cells was not detected, a finding that contrasts with the immunosuppression treatment with hyperfractionated total body irradiation (11 or 12 doses of 125 cGy for a total of 1,375 cGy or 1,500 cGy, at a dose rate of 8 to 20 cGy/min) and cyclophosphamide 60 mg/kg for 2 days. In pilot studies designed to improve the rate of engraftment, 12 patients received methylprednisolone in the early posttransplant period (5 mg/kg for 4 days beginning day +3 with tapering over 8 days) and nine patients received donor peripheral blood mononuclear cells, calculated to give 0.5 × 10^9/kg clonable T cells. These were infused with the marrow inoculum on day 0. Except for the 12 patients who received methylprednisolone, no patient received immunosuppression posttransplant for GVHD prophylaxis.

Four patients who died and four patients who relapsed before day 60 during the course of hematopoietic reconstitution could not be evaluated for engraftment. The evaluable patient population (114 patients) consisted of 57 patients with chronic myelogenous leukemia (CML), 35 patients with acute myelogenous leukemia (AML), and 22 patients with acute lymphoblastic leukemia (ALL). Nineteen of the 114 (17%) evaluable marrow graft recipients experienced graft failure.

Laboratory Assays

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque.

MATERIALS AND METHODS

Patient Characteristics

Patients in this study included 122 patients transplanted for leukemia at Memorial Sloan-Kettering Cancer Center. All patients in the study received a marrow transplant from an HLA identical sibling, which was depleted of T-lymphocytes by soybean agglutinin and sheep erythrocyte rosetting. All patients received pretransplant conditioning regimen and emerging at the time of rejection demonstrate antidonor reactivity and may thus play a role in graft failure. In particular, host T cells expressing the suppressor phenotype (CD3+, CD8+, Leu7+) that are capable of inhibiting donor hematopoiesis in vitro were detected in the peripheral blood of patients experiencing graft failure. Contrasting with our findings in patients who achieved durable engraftment, NK activity and NK cells were consistently absent from the peripheral blood of patients experiencing graft failure.

DEPLETION of T-lymphocytes from donor marrow can prevent graft-versus-host disease (GVHD) following allogeneic bone marrow transplantation (BMT). The technique of soybean lectin agglutination (SBA) followed by sheep erythrocyte (E) rosetting (SBA-E') has been particularly effective in removing donor T lymphocytes from a marrow graft prior to transplantation. Without additional posttransplant immunosuppression, no recipient of an HLA identical SBA-E' graft has experienced grade III-IV acute GVHD or chronic GVHD. Unfortunately, although the incidence and severity of GVHD have been markedly reduced, an increased incidence of graft failures has also been observed.

In an accompanying article, we have reported clinical observations derived from a study of 122 consecutive patients transplanted for leukemia with SBA-E' marrow from an HLA identical sibling. In this series 19 patients rejected their SBA-E' BMT. To investigate the basis for the graft failures observed, circulating peripheral blood mononuclear cells obtained at the time of graft failure have been characterized by phenotype and functional assays. Cytotoxic assays were performed with donor lymphoid and marrow targets as well as the natural killer (NK) cell target K562, and the ability of these cells to suppress marrow progenitor growth was assessed using cryopreserved donor marrow. The host effector T lymphocytes surviving the pretransplant conditioning regimen and emerging at the time of rejection demonstrate antidonor reactivity and may thus play a role in graft failure. In particular, host T cells expressing the suppressor phenotype (CD3+, CD8+, Leu7+) that are capable of inhibiting donor hematopoiesis in vitro were detected in the peripheral blood of patients experiencing graft failure. Contrasting with our findings in patients who achieved durable engraftment, NK activity and NK cells were consistently absent from the peripheral blood of patients experiencing graft failure.

MATERIALS AND METHODS

Patient Characteristics

Patients in this study included 122 patients transplanted for leukemia at Memorial Sloan-Kettering Cancer Center. All patients in the study received a marrow transplant from an HLA identical sibling, which was depleted of T-lymphocytes by soybean agglutinin and sheep erythrocyte rosetting. All patients received pretransplant conditioning regimen and emerging at the time of rejection demonstrate antidonor reactivity and may thus play a role in graft failure. In particular, host T cells expressing the suppressor phenotype (CD3+, CD8+, Leu7+) that are capable of inhibiting donor hematopoiesis in vitro were detected in the peripheral blood of patients experiencing graft failure. Contrasting with our findings in patients who achieved durable engraftment, NK activity and NK cells were consistently absent from the peripheral blood of patients experiencing graft failure.
graduates, as previously described. Peripheral blood lymphocytes (PBL) were obtained by depletion of monocytes by plastic adherence at 37°C. PBMC and/or PBL obtained from patients at the time of graft failure and from durably engrafted patients at different times in the posttransplant period were studied for donor-host origin, for cell-surface phenotype, and for antidonor reactivities.

Cytogenetic analysis. The donor and/or host origin of hematopoietic and lymphoid cells was established by cytogenetic analysis of marrow cells in spontaneous metaphase and phytohemagglutinin (PHA) stimulated circulating lymphocytes using autosomal polymorphisms determined by quinacrine banding in sex-matched patients and the Y chromosome in sex-disparate pairs.

Phenotype analysis. The cell surface phenotype of mononuclear cells isolated from the patients' peripheral blood was analyzed by two-color immunofluorescence using standard techniques. Directly fluorescein conjugated (FITC) HLe-1 (CD45), Leu-4 (CD3), Leu-3 (CD4), Leu-11 (CD16), Leu-7 (CD unassigned), HLA-DR, Leu-M3 (CD14), Ms1G1, and phycoerythrin-conjugated (PE) Leu-4, Leu-2 (CD8), Leu-19 (CD56), TAC (CD25) and Ms1G2 were purchased from Becton Dickinson (Mountain View, CA). Immunofluorescence samples were analyzed on an EPICS C cell sorter (Coulter Immunology, Hialeah, FL). The lymphoid populations analyzed were gated using log 90 light scatter and log forward angle scatter characteristics in conjunction with the leukocyte specific marker HLe-1 to eliminate RBC contamination. Residual monocyte contamination was evaluated by positive staining with the monocyte specific marker Leu-M3.

Colony inhibition assay (CIA). Inhibition of myeloid colony growth by peripheral blood mononuclear cells was assayed by a modification of the technique of Bordignon et al. Briefly, low density mononuclear cells were suspended in McCoy's 5A medium with 10% previously screened, heat-inactivated fetal calf serum (Hyclone, Logan, UT) and incubated with allogeneic bone marrow at an effector to bone marrow ratio of 2:1 and/or 1:1 for 18 hours at 37°C in humidified air containing 5% CO2. Triplicate cultures were plated in the standard colony forming unit granulocyte-macrophage (CFU-GM) assay according to the method of Pike and Robinson. Medium produced by the giant cell tumor (GCT-GIBCO) was used as a source of colony-stimulating factor (CSF). Bone marrow cells were resuspended (10^7/mL) in 0.3% agar in supplemented McCoy's medium, containing 10% CSF. The cells were cultured as described above and colonies (aggregates containing greater than 50 cells) were scored on day 14 of culture. The data are expressed as the percent inhibition of colony formation (CIA) compared with bone marrow plated alone calculated by:

\[
%\text{CIA} = \frac{\text{No. colonies control} - \text{no. colonies test}}{\text{No. colonies control}} \times 100
\]

Bone marrow mononuclear cells utilized for CIA experiments were obtained from the marrow donor at the time of the harvest of transplantation. Third party marrow was obtained from normal, consenting volunteers by multiple aspirations under local anesthesia. BM mononuclear cells were either Ficoll-Hypaque low density cells or SBA E+ marrow cells. BM cells were frozen in multiple vials containing 20 to 40 x 10^6 mononuclear cells in a modified cryopreservation solution containing 3% DMSO and 20% HIFCS. Because CIA experiments with either SBA E+ or low density marrow cells as targets gave similar results, the latter were selected for their superior growth after cryopreservation.

Chromium release assay. The cytolytic activities of freshly isolated peripheral blood mononuclear cells were measured in a standard 4-hour 51Cr release assay. The targets used were phytohemagglutinin (PHA) stimulated blasts derived from donor, host, and third party peripheral blood mononuclear cells, and the NK-sensitive cell line, K562. Effector cells were adjusted to concentrations that would yield effector to target (E:T) ratios of 100:1 or 50:1. Data were expressed as the percent specific lysis (SL) at a given E:T ratio calculated from the equation:

\[
\%\text{SL} = \frac{\text{CPM Experimental} - \text{CPM Spontaneous}}{\text{CPM Maximum} - \text{CPM Spontaneous}} \times 100
\]

Statistical analysis. The randomized model for paired comparisons test was utilized to detect a difference between anti-donor BM CIA and anti-unrelated BM CIA for the group of patients who experienced graft failure. The Wilcoxon two-sample rank-sum test was applied to examine differences between patients who engrafted and those who had graft failure with respect to anti-donor CIA and NK activities.

RESULTS

The clinical aspects associated with graft failure are extensively discussed in the accompanying report. Following transplantation, patients with durable engraftment showed prompt neutrophil recovery. Among patients whose graft failed, two patients failed to show any evidence of engraftment, four patients developed myeloid progenitors in the marrow with an increment in peripheral blood neutrophil counts that declined leaving only few circulating lymphocytes, and five patients developed early myeloid activity followed by an abrupt lymphocytosis between day 15 and day 20 posttransplant identical to the pattern we have described in patients who rejected HLA nonidentical T-cell-depleted marrow transplants. Eight patients reconstituted marrow cellularity and developed peripheral blood counts in a manner similar to those patients who achieved sustained engraftment. Between days 31 and 60, absolute peripheral blood neutrophil counts declined to less than 1,000/mm^3 and the patients developed marrow aplasia. No patient developed graft failure after day 60.

Functional analyses of the residual cells after graft failure, cytogenetic studies on their origin, and cell surface markers were performed at the first sign of graft failure. Unfortunately, in several of the later graft failures, this complication occurred after the patient had been discharged from the hospital; therefore, functional studies in this group often could not be performed at the appropriate time.

Origin of PMNC Isolated at the Time of Graft Failure

Circulating mononuclear cells isolated from the peripheral blood of these patients at the time of early graft failure were consistently of host type, as assessed by cytogenetic analysis of PHA stimulated peripheral blood lymphocytes (PBL). When no evaluable metaphases were obtained from freshly isolated PHA-stimulated PBL, T-cell lines were established from these cells. In 12 of 14 patients tested, interleukin-2 expanded T-cell lines derived from peripheral blood mononuclear cells were obtained at the time of graft failure. In each case 25 to 30 metaphases were examined and found to be 100% of host origin. In an additional patient, 80% of the cells were of host origin. However, in the other patient, 95% of the cells were of donor origin.
Cell Surface Phenotype of PBMC Isolated at the Time of Graft Failure

The phenotypic characterization of cell surface markers with two color immunofluorescence indicated that patients who achieved durable engraftment and hematopoietic recovery had circulating subpopulations of CD3+ T lymphocytes. However, the predominant cell population expressed the NK cell surface markers CD16 and Leu-19 (Fig 1). In contrast, PBMC from 12 patients (representative example; Fig 1) studied who were experiencing graft failure had predominantly CD3+ T cells (median of 88% with a range of 52% to 95%) of the CD8+ subset (median of 83% with a range of 35% to 87%). In each case NK cells were either absent or few in number (median of 0% with a range of 0% to 24%). Further analyses of these host-derived cells appearing at the time of graft failure indicated a preponderance of Leu-7+ CD8+ T lymphocytes. Additionally, a subpopulation of CD8+ cells were DR positive. Interestingly, the interleukin-2 receptor, TAC, was not detectable on the lymphocytes isolated from the peripheral blood of any of the patients experiencing graft failure (data not shown).

Functional Analysis of Host Lymphocytes

Functional assessments of host lymphocytes isolated at the time of graft failure from five recipients of HLA identical SBA-E marrow transplants revealed that PBMC from each of these patients had neither donor-specific nor nonspecific cytotoxic activity against peripheral blood targets in a 4-hour chromium release assay. This is in marked contrast to the donor-directed cytotoxicity observed in the HLA-nonidentical setting.14 Furthermore, in contrast to engrafted patients who recover NK activity as early as 14 to 18 days posttransplant,12 patients with graft failure had little or no NK activity (Fig 2). In this series the difference in the NK activity between those patients with durable engraftment and those with graft failure was highly significant (P < .01). One patient with graft failure had 20% kill of K562 and formed part of the rationale for increasing our fractionated total body irradiation from 1,375 to 1,500 cGy. Subsequently, normal NK cytotoxic activity was detected in only one patient with graft failure whose graft failure coincided with the development of a disseminated herpes zoster infection.

To examine the effect of peripheral blood mononuclear cells on the growth of donor marrow, coculture assays were performed. The host T-lymphocytes isolated from the peripheral blood of patients who experienced early graft failure exhibited strong colony inhibitory activity (CIA) specific for donor hematopoietic progenitors, when tested in vitro after an overnight coculture with donor, host or third party marrow, and subsequent seeding into the colony forming unit assay (day 14 CFU-GM) (Fig 3). When shorter coculture times were used, the inhibitory activity was less apparent; no inhibition was observed when effector cells were mixed with donor bone marrow immediately before seeding into the colony forming assay(s) (Fig 3). In all experiments in which the kinetics of the cellular interactions leading to colony inhibition were performed, a minimum time of coculture of 6 hours was required for subsequent detection of suppressive activity.

In contrast to these results, peripheral blood mononuclear cells, derived from engrafting patients at the same time in the posttransplant period, exhibited little or no inhibitory effect on the growth of donor or third party hematopoietic progenitors in the CIA assay (Fig 4). Based upon the Wilcoxon two-sample rank-sum test, in this series anti-donor CIA was significantly higher in patients who experienced early graft failure than in those who had prompt and sustained engraftment (P < .01).

The specificity of the host T lymphocytes emerging at the time of rejection was also examined by comparing colony growth following culture with donor or third party marrow progenitor in 10 patients with graft failure. The CIA observed was specifically directed against donor cells because inhibition of unrelated control bone marrow was significantly lower than that toward donor marrow (P < .02), as determined by the randomization model for paired comparisons.

The analysis of the risk factors for graft failure described in the companion report identified the donor sex (male) and age (older than 15 years) as variables for higher risk of graft failure. Accordingly, we have retrospectively analyzed our experimental groups for the possible influence of these two variables on test results. Sixteen of the 32 successfully engrafted patients whose T cells exhibited no colony inhibitory activity in vitro received marrow from male donors.

![Fig 1. Direct two-color immunofluorescence profile of PBMC isolated from the peripheral blood of a patient with durable engraftment (upper panel) and from a patient experiencing early graft failure (lower panel) after an HLA identical T-cell-depleted marrow transplant.](image-url)
Fig 2. NK activity as measured by percent specific lysis of K562 by PBMC isolated days 15 to 72 posttransplant from patients with durable engraftment (○), and from patients during the time of graft failure (●).

![Graph showing NK activity](image)

\( p < 0.01 \)

Fig 3. Time course for coculture of PBMC isolated at the time of graft failure with donor (○) or unrelated (●) bone marrow samples with subsequent seeding into the CFU-GM assay. The data are expressed as the percent inhibition of day 14 CFU-GM as compared with marrow plated in the absence of effector cells.

![Graph showing time course](image)

Fig 4. Colony inhibitory activity of PBMC isolated days 15 to 72 the posttransplant period from patients with durable engraftment (engraft), and from patients during the time of graft failure (GF). (○). Antidonor-specific activity. (●) Colony inhibitory activity generated against unrelated bone marrow cells.

![Graph showing colony inhibitory activity](image)

Additionally, the patient age was greater than 15 years in 24 of the 32 engrafted recipients studied. In the graft failure group, 9 of 10 patients studied had a male donor. In our in vitro analysis, the unrelated marrow samples were from a male donor in 4 of 10 cases. Inhibition of colony growth was similarly low when cells from either male or female third party donors were used. In this group no marrow sample utilized was from a donor younger than 15 years.

**DISCUSSION**

Graft failure in recipients of an unmodified bone marrow transplant from an HLA identical sibling has been almost exclusively limited to patients transplanted for aplastic anemia who have a history of repeated transfusions. In leukemic patients the incidence of graft failure for unmodified marrow transplants is less than 1%. However, graft failure has been a relatively frequent occurrence in virtually every study in which marrow transplants depleted of T lymphocytes have been used for prevention of GVHD. This has been a consistent observation that is independent of the method employed for depletion.
Bone marrow manipulations associated with the different techniques utilized for T-cell depletion could be responsible for graft failure through a number of different mechanisms, including loss of stem cells, elimination of lymphoid and nonlymphoid cells with accessory functions for hematopoietic development, and removal of T cells capable of down-regulating residual host effectors responsible for graft rejection. Even though it is possible that all these mechanisms contribute to some extent to graft failure, there is considerable experimental and clinical evidence suggesting that the first two possibilities do not play a primary role. In a separate study, graft failure did not occur in any of the patients who received autologous marrow depleted of T cells by the same methods utilized for allogeneic transplantation. In contrast, the incidence of graft failure in the allogeneic group was 27%.24 Rather, residual host resistance is believed to be a prominent mechanism responsible for graft rejection.25

Resistance to hematopoietic grafts contributing to graft failure has been most extensively characterized in murine and canine models, in which at least two mechanisms have been distinguished. One reflects an unprimed host resistance to engraftment26-28 and the other a rejection secondary to prior sensitization to donor antigens.29-31 Lethally irradiated dogs and mice, previously sensitized to MHC-matched or mismatched allogeneic donors through infusions of blood or marrow cells, reject marrow grafts by what is presumed to be a specific immune response.29,30 In the mouse Cantor31 demonstrated that cytotoxic T cells generated in vitro in response to major or minor alloantigens are capable of suppressing hematopoietic cell growth in vivo. Additionally, the distinct contribution of T-lymphocytes to rejection of bone marrow allografts has been directly demonstrated by Dennert et al.32 Similarly, Torok-Storb et al33 have shown that canine lymphocytes sensitized to minor alloantigens fail to support the growth of donor erythroid progenitors in vitro, thus predicting rejection of DLA-identical marrow grafts.

After an HLA nonidentical T-cell–depleted transplant, leukemic patients who have experienced graft failure have circulating host T-lymphocytes with proliferative34 and cytotoxic activity35 directed toward donor peripheral blood targets. Sondel et al36 demonstrated that PBMC obtained from a recipient of a D mismatched marrow had a selective proliferative response against donor cells in a mixed lymphocyte culture reaction, with kinetic patterns suggesting a secondary response, as observed in in vitro primed lymphocyte testing. Our own studies demonstrated that rejection of T-cell–depleted HLA nonidentical marrow grafts was associated with the emergence of host-derived T-lymphocytes predominantly of the CD8+, cytotoxic T-cell subset.14 These PBMC exhibited antidonor, cell-mediated cytotoxicity when tested in vitro. Furthermore, in one patient the cytotoxicity was shown to be directed against a class I (B7) sensitizing antigen present on the donor lymphocytes. Consistent with these findings, the circulating CD8+ cells are Leu 7- by phenotypic analysis.

In HLA identical combinations, Bunjes et al14 identified host-derived CD8+ T-cells in three leukemic patients with graft failure following a T-cell–depleted marrow transplant. In our studies recipients of HLA-matched SBA–E– marrow grafts who achieved durable engraftment developed a mixture of T cells and NK cells. In contrast, circulating lymphocytes from patients who experienced graft failure were exclusively T cells of host origin, which were of the CD3+, CD8+, Leu7+, Leu19- phenotype. These host T cells were not cytotoxic against donor targets but were able to inhibit the growth of donor bone marrow colony forming units in vitro. These functional features are consistent with their phenotype in that these CD8+ host T cells were largely Leu7+, with a prominent DR+ subset. Although three-color immunofluorescence was not performed, the data suggest the presence of CD8+, Leu 7+, DR + lymphocytes, a subpopulation normally detected at low frequency in normal adult bone marrow37 and peripheral blood,38 which is known to have marrow suppressive activity35,37 and may be an important regulator of hematopoiesis.39 In the setting of marrow transplantation, it is possible that host cells of this subset contribute to graft failure following transplantation of a T-cell–depleted graft. In our study the emergence of these host T lymphocytes of the suppressor phenotype was a consistent feature in all the patients who failed their graft, independent of the sex of the donor, age of recipient, the history of sensitization, or the pattern of graft failure observed. Specific antidonor colony inhibitory activity was consistently observed only in those patients who failed their graft early in the posttransplant period. Inhibition of CFU-GM from third party donors, even male donors, was low or not detected. In our series SBA–E– marrow grafts from male donors have been found to be at particular risk for failure or rejection. One possibility that might in part explain this finding is that the H-Y antigen is targeted by residual host T cells, as has been suggested by Goulmy et al.38 The failure to detect inhibition of the growth in these studies is not inconsistent with the possibility because such H-Y specific responses are HLA restricted.38,40 Additional studies are in progress to determine whether in certain cases specific inhibition of donor CFU-GM can indeed be ascribed to HLA restricted H-Y specific reactivity.

Six of the graft failures occurred after the patients had been discharged from the transplant unit; therefore, they could not be tested in the early stages of graft failure. It is thus possible that these later graft failures reflect host resistance mechanisms identical to those detected in patients with early graft failure. However, we cannot discard the possibility that a proportion of these failures are indeed of different etiology, such as infection, other effector systems, or defects in the host stromal environment.

The absence of natural killer cells and activity in the peripheral blood of those patients who experienced graft failure and the consistent predominance of lymphocytes with the NK phenotype early in the posttransplant course of patients who achieved durable engraftment suggest that these effector cells are not involved in the acute phase of rejection. Indeed, the absence of NK cells and activity early posttransplant can be interpreted as an early sign of graft failure.

Further studies of the role played by these host T-lymphocytes emerging in patients who suffer graft failure
following an allogeneic T-depleted graft and the mechanisms by which graft failure occurs may allow the design of more specific and effective protocols to overcome graft resistance in patients transplanted for leukemia.

ACKNOWLEDGMENT

The authors thank Teresa Cartagena for her technical support and Carolynn Barnes for her help in preparing the manuscript.

REFERENCES


7. Olson SB, Magenis RE, Lovrien EW: The discriminatory power of Q-band Heterochromatin (variant) analysis in distinguishing between individuals with specific application to cases of questionable paternity. Am J Hum Genet 38:235, 1986


Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukemia: II. In vitro analyses of host effector mechanisms

C Bordignon, CA Keever, TN Small, N Flomenberg, B Dupont, RJ O'Reilly and NA Kernan