Prediction of Graft-Versus-Host Disease by Phenotypic Analysis of Early Immune Reconstitution After CD6-Depleted Allogeneic Bone Marrow Transplantation

By Robert J. Soiffer, René Goinin, Christine Murray, Michael J. Robertson, Keith Cochran, Steven Chartier, Christine Cameron, John Dalay, Herbert Levine, Lee M. Nadler, and Jerome Ritz

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality following allogeneic bone marrow transplantation (BMT). Because GVHD is frequently refractory to treatment, the early identification of high-risk patients could have significant clinical value. To identify such patients, we examined early immunologic reconstitution in 136 patients with hematologic malignancies who received anti-T12 (CD6)-purged allogeneic bone marrow over a 9-year period. The majority of patients received marrow from HLA-matched sibling donors after ablation with cyclophosphamide and total body irradiation. No patients received any immune suppressive medications for GVHD prophylaxis. The fraction and absolute numbers of peripheral blood lymphocytes (PBL) expressing the CD3, CD4, CD8, and CD56 surface antigens were determined weekly by immunofluorescence analysis in patients beginning 8 to 14 days (week 2) after marrow infusion. Results in patients who did or did not subsequently develop GVHD post-BMT were compared. Within 2 weeks of marrow infusion, patients who developed grades 2-4 GVHD had significantly higher percentages and absolute numbers of CD8+ T cells and a lower fraction of CD56+ natural killer (NK) cells than individuals who remained free of GVHD. Thirty-five percent of patients whose PBL were greater than 25% CD8+ in the second posttransplant week developed GVHD, compared with only 3% of patients who had ≤25% CD8+ cells (odds ratio 37.8; 95% confidence interval [CI] 4.1 to 397). A subgroup of patients at very high risk for GVHD could be identified based on the combined frequency of CD8+ T cells and NK cells in blood. Seventy-five percent of patients with greater than 25% CD8+ cells and ≤45% CD56+ cells during week 2 post-BMT developed GVHD, compared with only 11% of the remaining patients (odds ratio 24.9: 95% CI, 5.3 to 117.0). None of the 23 patients with both less than 25% CD8+ cells and greater than 45% CD56+ cells in the second posttransplant week developed grades 2-4 GVHD. Our findings indicate that CD8+ T cells play an important role in the pathogenesis of GVHD in humans. Analysis of immune reconstitution early after BMT is useful in predicting the onset of GVHD and can help direct the implementation of treatment strategies before the appearance of clinical manifestations. Such interventions may decrease the morbidity and mortality associated with allogeneic BMT and ultimately improve overall survival.

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PREDICTION OF GVHD

moreover, data from animal and human studies have correlated the number of T lymphocytes infused with donor marrow with the subsequent likelihood of developing GVHD.16

As early as 10 to 14 days after marrow infusion, the peripheral blood (PB) of patients who subsequently went on to have clinically significant GVHD (grades 2-4) contained a higher number and percentage of CD8\(^+\) T cells and a lower fraction of CD56\(^+\) natural killer (NK) cells than did the PB of individuals who remained free of GVHD. Our findings support the role of CD8\(^+\) T cells in the pathogenesis of GVHD in humans and suggest that flow cytometric analysis of early reconstituting cells can be used to predict the onset of GVHD before the appearance of clinical manifestations.

MATERIALS AND METHODS

Patient characteristics. Between August 1983 and February 1992, 148 consecutive adult patients underwent allogeneic BMT on a single Dana-Farber Cancer Institute (DFCI, Boston, MA) protocol.17 Immunofluorescence studies of PB mononuclear cells (PBMC) during the first 6 weeks post-BMT were performed and available on 136 engrafting patients. Nine patients (3 of 131 patients receiving HLA-matched sibling grafts and 6 of 17 patients receiving donor marrow with the subsequent likelihood of developing grades 2-4 GVHD) were excluded from analysis. Data was unavailable on 3 additional patients, none of whom had developed GVHD. Among the study population, the median age was 36 years (range, 17 to 59 years) at the time of BMT; there were 81 men and 55 women. Diagnoses included chronic myelogenous leukemia (CML; n = 47), acute myelogenous leukemia (AML; n = 35), acute lymphoblastic leukemia (ALL; n = 18), Hodgkin’s and non-Hodgkin’s lymphoma (n = 12), multiple myeloma (n = 12), myelodysplastic syndrome (n = 6), and chronic lymphocytic leukemia (n = 6). One hundred twenty-six patients received marrow from HLA-identical sibling donors, whereas 10 patients received partially HLA-mismatched grafts from related donors.

Treatment protocol. The majority of patients received cyclophosphamide (60 mg/kg intravenously [IV] \(\times\) 2) followed by 1,400 cGy TBI as their ablative regimen. Nine patients who received prior radiotherapy were treated with busulphan (16 mg/kg) instead of TBI. For the majority of patients, TBI was administered in 7 equal 200-cGy fractions over 4 days.17 Since 1986, patients with CML received additional radiation therapy (600 to 750 cGy) to the spleen just before the transplant-preparative regimen. Patients who received grafts that were not HLA genotypically identical also received total lymphoid irradiation (600 to 1,050 cGy) 1 to 2 weeks before admission.18 All patients received fresh donor marrow that was treated in vitro with anti-T12 (CD6) monoclonal antibody (MoAb) and rabbit complement to remove mature T lymphocytes.19 T12 depletion of donor marrow was the sole form of GVHD prophylaxis used in our patients. No patients received prophylactic immunosuppressive therapy of any type either before or after marrow infusion. All patients were treated using standard reverse isolation procedures. Oral prophylactic antibiotics, either ciprofloxacin or trimethoprim-sulfamethoxazole, were administered to all patients. These were discontinued when patients developed fevers that required initiation of broad-spectrum intravenous antibiotics. Since 1986, all patients have received prophylactic acyclovir. All patients received single-donor platelets from CMV-seronegative individuals and frozen deglycerolized red blood cells. All blood components were irradiated (20 Gy) to prevent transfusion-related GVHD. The diagnosis of acute GVHD was made clinically and confirmed histologically in the majority of cases. Grading of GVHD was based on criteria previously established.19 Corticosteroids were instituted as the first line of therapy for patients with grades 2-4 GVHD. The treatment protocol was approved by the Human Subjects Protection Committee of DFCI and written informed consent was obtained in all cases.

Immunophenotypic analysis. Blood samples were obtained weekly, if possible, beginning 10 to 14 days after marrow infusion. PB lymphocytes were isolated using Ficoll-Hypaque density centrifugation and were analyzed for reactivity with a series of MoAbs using standard techniques. Single-color and dual-color immunofluorescence assays were performed as previously described.20 Antibodies used included T3 (CD3), T4 (CD4), T8 (CD8), T12 (CD6), and NKH1 (CD56) (Coulter Immunology, Hialeah, FL). Ten thousand cells falling within the lymphocyte gate were analyzed in each sample using automated flow cytometry (EPICS-V; Coulter Electronics, Hialeah, FL). Samples were available on 93 patients at week 2, 110 patients at week 3, 93 patients at week 4, 63 patients at week 5, and 51 patients at week 6.

Statistical analysis. Descriptive statistics are reported as proportions, means, and medians. The percentage and absolute number of PBMC expressing specific surface antigens (CD3, CD4, CD6, CD8, and CD56) were aggregated for each patient over weekly intervals beginning 8 to 14 days (week 2) post-BMT through day 42 (week 6). In the initial exploratory analysis, the median values of CD3, CD4, CD6, and CD56 for each week were compared in patients with clinically significant GVHD (grades 2-4) and in those with minimal or no GVHD (grades 0-1) by the Mann-Whitney U test.21 Patients with grades 0 and 1 GVHD were considered together because of the similarity of their clinical courses as well as the similarity of their observed immunologic reconstitution. Differences in the mean percent values of CD3, CD4, CD8, and CD56 were tested for significance by examining the log-likelihood ratio statistic. Pre-BMT prognostic variables were also considered in a multiple logistic regression analysis using stepwise selection.23 Immunophenotypic data for all surface antigens (CD3, CD4, CD6, CD8, and CD56) included in this regression analysis were available on 77 patients during week 2 post-BMT.

Pretransplant clinical variables were assessed to determine if they influenced the subsequent development of GVHD. These variables included HLA match, patient age, donor age, patient sex, donor sex, diagnosis for which BMT was performed, stage at the time of BMT, CMV seropositivity, and Herpes simplex virus (HSV) seropositivity. The number of T cells infused in the marrow inocula was also analyzed. For the categorical variables, associations between risk factors and GVHD outcome were tested by means of Fisher’s exact test.21 The association between continuous predictors were tested for significance by examining the log-likelihood ratio \(\chi^2\) statistic.23 Pre-BMT prognostic variables were also considered in a multiple logistic regression analysis using stepwise selection.23 A subsequent multiple logistic regression analysis, including both the pertinent immunophenotypic variables (CD8 and CD56) as well as the most relevant pretransplant variables as determined above, was performed. The inclusion of these pretransplant factors was found not to improve the overall model. The odds ratios for developing GVHD and 95% confidence intervals (CI) were calculated for each variable.

Dichotomous subgroups for the phenotypic variables (CD8 and CD56) that had been found to be independently associated with the
development of grades 2-4 GVHD by multiple logistic regression analysis were created based on regression tree models (recursive partitioning). The cutpoints selected by this method (24.5% for CD8 and 44.5% for CD56) were close to the respective median percentages for these phenotypic variables. For subsequent analyses, cutpoints of 25% for CD8 and 45% for CD56 were used. When absolute numbers of cells were evaluated, cutpoints of 0.030 x 10^9 cells/L for CD8 and 0.100 x 10^9 cells/L for CD56 were used. Differences in the proportion of patients from dichotomous phenotypic subgroups who developed GVHD were compared by Fisher's exact test. A model to further define the likelihood of developing grade 2-4 GVHD using these dichotomous subgroups was constructed. Patients were divided into groups based on whether their PBMC contained greater or less than 25% CD8+ cells and greater or less than 45% CD56+ cells. A similar analysis was performed using the absolute number of CD8+ and CD56+ cells. The likelihood of developing GVHD in the group of patients with the highest incidence of GVHD was compared with that in the remaining patients by calculating the log-odds ratio. Time to GVHD (risk) curves for these two groups were constructed by means of the Kaplan-Meier product limit method. The logrank test of survival analysis was also used to compare patients at high risk for developing GVHD (CD8 >25% and CD56 ≤45%) with all others with respect to their time to GVHD distribution.

**RESULTS**

*Incidence of GVHD.* Within the cohort of 136 patients under consideration, acute GVHD (grades 2-4) was diagnosed in 26 patients (19%; 95% CI, 13% to 27%) in 21 of 126 (17%) patients receiving HLA-matched, mixed lymphocyte culture (MLC)-nonreactive sibling grafts; and in 5 of 10 (50%) patients receiving partially HLA-mismatched marrow. The initial appearance of clinical signs of GVHD occurred at a median of 25 days post-BMT (interquartile range, 18 to 28 days). In all cases, corticosteroids were used initially to treat patients when signs and symptoms escalated. Systemic steroids were begun at a median of 34 days post-BMT (interquartile range, 24 to 43 days) in patients with grades 2-4 GVHD. Of the 26 cases of GVHD, 16 were grade 2 and 10 were grade 3 or 4. Eight patients progressed to develop chronic GVHD. Transplant-related mortality was higher in patients with grades 2-4 acute GVHD, occurring in 12 of 26 (46%) cases compared with only 15 of 110 (14%) patients with grades 0-1 GVHD (Fisher's exact test, P = .0006). The mortality rate in patients with grade 0 GVHD was virtually identical to that in patients with grade 1 GVHD (13.0% v 14.5%, P = NS).

**Pre-BMT clinical variables and GVHD.** Before performing the analysis of immunophenotypic recovery post-BMT, we evaluated the influence of a number of pretransplant clinical variables on the development of GVHD. For this analysis, we included factors that have been suggested by others to be important, including degree of HLA match, patient age, donor age, patient sex, donor sex, the number of T cells infused with the marrow inocula, diagnosis for which BMT was performed, stage of disease at the time of BMT, CMV seropositivity, and HSV seropositivity. Table 1 presents the results of a univariate analysis of these variables in all 136 patients. A genotypically non-HLA identical marrow donor source was the only factor associated with the development of grade 2-4 GVHD in recipients of CD6-depleted marrow (Fisher's exact, P = .02).

**Immunophenotypic analysis.** We compared immunologic recovery during the first 6 weeks after CD6-depleted allogeneic BMT in patients who did and did not go on to develop grades 2-4 GVHD. Results were grouped by week post-BMT so that samples obtained 8 to 14 days after marrow infusion were grouped in week 2, 15 to 21 days in week 3, etc. As early as week 2 post-BMT, significant differences in the distribution of lymphoid subsets were evident. Patients who subsequently developed GVHD had a greater fraction of CD3+ T cells (Mann-Whitney U test, P < .005) and a lower fraction of CD56+ NK cells (P < .02) in their PB than individuals who never developed grade 0 or 1 GVHD (Fig 1). The phenotypic composition of patients with grade 0 (no) GVHD and grade 1 (mild) GVHD was identical (data not shown). The increased percentage of T cells in patients with GVHD was caused by an increase in the fraction of CD8+ cells in patients with GVHD (P < .001). No significant difference in the percentage of CD4+ cells was noted between these two groups. When absolute numbers of CD3+ or CD8+ T cells were examined, significant differences were again noted (data not shown). No difference in the distribution of the CD8 surface antigen on CD56+ NK cells was

### Table 1. Pre-BMT Variables and the Incidence of GVHD

<table>
<thead>
<tr>
<th>Clinical Variable</th>
<th>Incidence of GVHD</th>
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<tr>
<td>HLA match</td>
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<tr>
<td>No</td>
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<tr>
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<tr>
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<td></td>
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<tr>
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<tr>
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<td>(continuous variable)</td>
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* Calculated by Fisher's exact test for categorical and by log-likelihood ratio χ^2 for continuous variables.

† Early stage includes acute leukemia in first remission and chronic phase CML.
observed in patients with and without GVHD as determined by dual-color immunofluorescence.

In the majority of cases, the striking difference in immunophenotypic profiles in patients with grades 0-1 and grades 2-4 GVHD was seen at a time before any clinical signs of GVHD were evident. The initial immunophenotypic analyses in these patients were performed less than 2 weeks post-BMT, when the median white blood cell count was only $0.2 \times 10^9$ cells/L (at days 11 through 13 post-BMT) and when the median absolute lymphocyte count was $0.038 \times 10^9$ cells/L. As shown in Fig 1, the higher mean frequencies of CD3+ and CD8+ T cells in patients who developed GVHD were noted not only during week 2 post-BMT but also in subsequent weeks as well (repeated measures ANOVA, $P < .01$). However, by 6 weeks post-BMT, when GVHD was clinically evident in most cases and already treated in many, differences in immunophenotypic profiles could no longer be found.

Using two-color immunofluorescence staining, we also examined T cells for expression of the CD6 antigen, the target of our purging process. We have previously shown that, although greater than 95% of T cells from normal volunteers have detectable surface CD6, less than 70% of T cells from patients early post-BMT express this antigen.$^{37}$ T

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**Fig 1.** Surface antigenic frequencies on PBMC from patients over time after CD6-depleted allogeneic BMT. The frequencies of (A) CD3, (B) CD56, (C) CD8, and (D) CD4 surface antigens are displayed over the first 6 weeks post-BMT in patients with grades 0-1 GVHD (square) and grades 2-4 GVHD (circle). Data points represent mean values from samples obtained from greater than 90 patients during weeks 2 through 4 and 55 patients from weeks 5 and 6.
cells that lack CD6 on their surface can persist in the circulation for several years post-BMT. As shown in Fig 2, there were more CD6+ cells within the first 2 weeks of BMT in patients who subsequently developed GVHD than in those in whom GVHD did not occur (Mann-Whitney U test, \( P < .01 \)). In contrast, there was no difference in the frequency of CD6- cells between these two groups.

**Immunophenotypic models and risk of GVHD.** The early detection of accelerated T-cell reconstitution in individuals who developed GVHD led us to examine the predictive value of specific phenotypic profiles. We again focused on week 2 posttransplant, because clinical signs had not yet appeared at this point in the majority of patients who developed GVHD. A univariate analysis of CD3, CD4, CD8, and CD56 immunophenotypic frequencies confirmed that a high fraction of CD3+ and CD8+ T cells as well as a low percentage of CD56+ NK cells were associated with the subsequent onset of GVHD. These three phenotypic variables were then considered in a multiple logistic regression analysis using stepwise selection on the 77 patients for whom values for all three variables were available during week 2 post-BMT. Only CD8 (likelihood ratio \( \chi^2, P < .005 \)) and CD56 (likelihood ratio \( \chi^2, P < .04 \)) maintained their significance in this analysis.

In an attempt to increase the informative value of our immunophenotypic determinations, we next created dichotomous subgroups for patients with high and low CD8 and CD56 values using recursive partitioning. Patients were separated into those whose PBMC contained greater than 25% CD8+ cells and those with \( \leq 25\% \) CD8+ in week 2 post-BMT. As shown in Table 2, 35% (15 of 43) of patients who had greater than 25% CD8+ cells in their PB went on to develop grade 2-4 GVHD compared with only 3% (1 of 34) of those with \( \leq 25\% \) CD8+ cells (Fisher’s exact, \( P = .0005 \)). When absolute numbers of CD8+ cells were evaluated, striking differences were again observed. Patients were divided into those with greater than 0.030 \( \times 10^9 \) CD8+ cells/mL and those with \( \leq 0.030 \times 10^9 \) CD8+ cells/mL at the time phenotypic measurements were performed during week 2 posttransplant. Of 46 patients with greater than 0.030 \( \times 10^9 \) CD8+ cells/mL, 15 (33%) developed GVHD, compared with only 1 of 30 (3%) patients with fewer CD8+ cells (Fisher’s exact, \( P = .003 \)). A similar analysis was performed for the NK cell marker CD56. When PB contained \( \leq 45\% \) CD56+ cells, 43% (10 of 23) of the patients subsequently developed GVHD, compared with only 11% (6 of 54) of individuals with greater than 45% CD56+ cells (Fisher’s exact, \( P = .004 \)). We next incorporated the pretransplant clinical factors evaluated earlier, including HLA disparity between donor and recipient, into a single stepwise logistic regression analysis along with these immunophenotypic variables. When these factors were all considered together, only a CD8 value greater than 25% (odds ratio 37.8; 95% CI, 4.1 to 397) and a CD56 value \( \leq 45\% \) (odds ratio 13.7; 95% CI, 3.0 to 66) emerged as significant predictors of GVHD.

To identify a group of patients who might be at very high risk of developing GVHD, we constructed a model that combined information obtained from the analysis of CD8 and CD56 antigen frequencies. Table 3 displays the incidence of GVHD in four groups of patients separated on the basis of the frequency of CD8+ and CD56+ cells in their PB. In all, 75% of patients whose PBMC contained greater than 25% CD8+ and \( \leq 45\% \) CD56+ cells in week 2 post-BMT went on to develop grades 2-4 GVHD, compared with only 11% of the remaining patients (odds ratio 24.9; 95% CI, 5.3 to 117.0). Figure 3 shows the difference in the time to GVHD in these two groups (logrank, \( P < .001 \)). The usefulness of combining both the CD8 and CD56 immunophenotypic parameters in these analyses is underscored by the fact that, in patients with an elevated proportion of CD8+ cells (\( > 25\% \)) but in whom greater than 45% of PBMC were CD56+, only 19% developed GVHD (Table 3). As has been previously stated, those individuals whose PBMC contained a low percentage (\( \leq 25\% \)) of CD8+ cells had a very low likelihood of developing GVHD. Indeed, no patients whose PBMC were simultaneously \( \leq 25\% \) CD8+ and greater than 25% CD56+ developed grade 2-4 GVHD compared with only 3% (1 of 34) of those with \( \leq 25\% \) CD8+ cells (Fisher’s exact, \( P = .0005 \)).
45% CD56+ were diagnosed with GVHD. Again, when absolute numbers of CD8 and CD56 cells were combined into a similar model, a group of patients at very high risk for imminent GVHD could be identified. Specifically, patients whose PB contained greater than $0.030 \times 10^9$ CD8+ cells/mL and less than $0.100 \times 10^9$ CD56+ cells/mL were far more likely to develop GVHD compared with the remaining patients (60% v 7%; odds ratio 19.5; 95% CI, 5.3 to 77). Thus, determining the immunophenotypic profile in this manner can help separate patients into those with a very high or very low risk of subsequent GVHD.

### DISCUSSION

In this report, we present our observations on early immunologic recovery following allogeneic marrow transplantation and its influence on the subsequent development of GVHD. At a time before the appearance of clinical signs of GVHD, the lymphoid composition of patients who ultimately developed GVHD was significantly different from that of patients who never developed GVHD. Specifically, patients developing GVHD had a greater fraction of CD3+ CD8+ T cells and a lower fraction of CD56+ NK cells than their unaffected counterparts. Moreover, we could construct a model based on the percentage or number of PBMC expressing CD8 and CD56 that could identify subgroups of patients with a very high (>70%) and a very low risk of subsequent GVHD.

Few extensive studies on the immunophenotypic characterization of lymphocytes in PB have been conducted in patients with acute GVHD.\textsuperscript{28-31} The role of CD8+ T cells has been suggested by several histopathologic studies on the skin and gut of patients with acute GVHD.\textsuperscript{10,11,12} However, results have not always been clear cut, with some investigators suggesting that CD4+ T cells or NK cells can function as effectors of GVHD.\textsuperscript{10,33-35} Nonetheless, reports of the partial prevention of GVHD by in vitro marrow purging with anti-CD8 antibodies supports the contention that CD8+ cells play a critical role in human GVHD.\textsuperscript{36} Indeed, in our series, only 3% of patients whose PBMC contained less than 25% CD8+ cells in week 2 post-BMT later developed grade 2-4 GVHD. Although CD8 is expressed on a certain fraction of NK cells, dual-color immunofluorescence studies confirm that the association between the appearance of CD8+ cells and GVHD is accounted for by CD8+ T cells, not NK cells (data not shown).

These observations further underscore the importance of CD8+ T cells in GVHD. The increased fraction of CD8+ T cells we noted in our patients with GVHD may represent a direct response of the engrafting donor marrow to newly recognized minor host antigens. It is uncertain whether these CD8+ cells initiate the clinical changes of GVHD directly or by inducing the secretion of cytokines (tumor necrosis factor-$\alpha$, interleukin-1$\beta$, etc) that mediate tissue damage.\textsuperscript{37,38} Although we suspect that the CD8+ cells themselves are pivotal in the pathogenesis of GVHD, it is possible that the early expansion of CD8+ T cells in patients with GVHD is the result of a biologic response to cytokines elaborated by another cell type.\textsuperscript{40}

Dual-color immunofluorescence studies suggest an association between the number of CD6+ but not CD6- T cells and the onset of GVHD. The emergence of CD6+ T cells early after marrow infusion in patients who developed GVHD may indicate that the purging process did not remove a sufficient number of CD6+ T cells from donor marrow to prevent GVHD. However, immunophenotypic analysis of postpurging marrow samples has not shown any association between residual CD6+ cells in the marrow and subsequent GVHD,\textsuperscript{17} although it is possible that our methods for detection of residual CD6+ cells in the marrow were not sufficiently sensitive. Alternatively, the association between CD6+ T cells and GVHD may depend not on the adequacy of purging but rather on the differential response
of CD6+ and CD6− T cells to host-antigenic stimuli. Indeed, we have preliminary evidence that CD6+ T cells are not as responsive as CD6− T cells to a variety of stimuli in vitro (data not shown).

Our results show that the analysis of immune reconstitution by flow cytometry is very useful in predicting the development of GVHD in patients undergoing CD6-depleted allogeneic BMT. It is uncertain whether similar observations can be made in recipients of unmanipulated marrow. However, it is clear that valuable immunologic information can be obtained from PB within 2 weeks of BMT. The importance of identifying patients destined to develop GVHD is underscored by the observation that nonrelapse mortality rates are much higher for these patients than for those individuals without GVHD. Patients identified by flow cytometry to be at high risk for GVHD may become candidates for trials of early transplantation. In addition, because it has been suggested that the absence of GVHD after BMT may be associated with a higher risk of disease recurrence, identification of individuals who have a low likelihood of developing GVHD can help in the selection of patients for trials of early treatment with immunomodulatory cytokines designed to induce graft-versus-leukemia activity.41 The impact of the analysis of early immune reconstitution on disease-free survival post-BMT must now be evaluated.

REFERENCES


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