T-Lymphocyte Reconstitution in Recipients of Bone Marrow Transplants With and Without GVHD: Imbalances of T-Cell Subpopulations Having Unique Regulatory and Cognitive Functions

By Wilhelm Friedrich, Richard J. O'Reilly, Benjamin Koziner, David F. Gebhard, Jr., Robert A. Good, and Robert L. Evans

The development of T lymphocytes in 34 patients engrafted with allogeneic bone marrow was studied by indirect immunofluorescence using mouse monoclonal antibodies. Two antibodies, termed anti-Leu-1 and anti-Leu-4, react with distinct membrane components expressed by all peripheral T cells, whereas two other antibodies, termed anti-Leu-2a and anti-Leu-3a, react with complementary subsets of Leu-1+ and Leu-4+ lymphocytes. The Leu-2a+3a− (Leu-2) subset contains suppressor/cytotoxic T cells, and the Leu-2a−3a+ (Leu-3) subset contains helper T cells. The ratio of Leu-2/Leu-3 T cells was elevated in nearly all patients studied during the first month of engraftment, but this ratio subsequently returned to normal in patients with an uncomplicated course or with solved acute GVHD. In contrast, a deficiency of helper T cells with a resultant preponderance of suppressor/cytotoxic T cells persisted in all patients studied with both acute and chronic GVHD after the first month. The implications of these findings in regard to the functions of these cells in vitro are discussed.

The major limitation to the clinical application of marrow allografts for the treatment of lethal blood disorders is the failure to adequately control graft-versus-host disease (GVHD). Although the development of allogeneic T cells is widely accepted to be essential to the initiation of GVHD, the cellular interactions leading to the pathologic expression and frequent resolution of this disease remain largely unknown. Evidence from certain rodent models has suggested that tolerance of the graft for the host is an active process, whereby cells apparently promoting tolerance in vivo have suppressor activities in vitro1,3 Other studies that have characterized the functional heterogeneity of T lymphocytes in the mouse4,5 and more recently in man6−8 would also suggest that the expression of GVHD and the induction of tolerance of the graft for the host is at least partially determined by the regulatory interactions between two major functional subpopulations of T cells.

To study these two T-cell subsets, we recently produced a panel of monoclonal antibodies by somatic hybridization techniques9 that are directed against distinct T-cell surface antigens.10,12 One of these membrane antigens is restricted to T cells having cytotoxic/suppressor functions (herein termed Leu-2 T cells), and another is restricted to T cells having helper/inducer functions (Leu-3 T cells).6,8 These markers were used to examine the progressive development of both T-cell subsets in the peripheral blood of 34 recipients of marrow transplants, of whom 21 had acute GVHD and 10 had chronic GVHD during the period of study. Our findings are in some regards consistent with a prior study suggesting that suppressor/cytotoxic T cells emerge as the numerically dominant subset in the majority of patients with chronic GVHD,13 but differ from that study in regard to the T-cell subset imbalances seen in patients with acute GVHD.

MATERIALS AND METHODS

Patients

Studies were performed prospectively on 34 patients following marrow transplantation for acute leukemia (30 patients) or aplastic anemia (4 patients). HLA-A,B,C,D matched siblings served as donors for 32 patients; 2 patients received transplants from HLA-A,B,C nonidentical but HLA-D-compatible parents. Informed consent was obtained from each patient or the responsible parent or guardian. Consenting healthy laboratory personnel served as blood donors for control studies.

Patients with acute leukemia were prepared for transplantation with 1320 rad fractionated total body irradiation (TBI) administered in 11 doses of 120 rad over 4 days followed by cyclophosphamide (60 mg/kg for 2 days) as previously described.14 Two patients with aplastic anemia were prepared with cyclophosphamide (50 mg/kg/day for 4 days), 6-thioguanine (200 mg/kg/day for 5 days), and 6-thioguanine (200 mg/kg/day for 5 days); the other two patients received fractionated TBI (200 rad/day for 4 days) followed by cyclophosphamide (60 mg/kg/day for 2 days). All patients received methotrexate prophylaxis posttransplant.15

Acute graft-versus-host disease was diagnosed and graded on the basis of clinical findings according to the criteria of Glucksberg et al.16 The diagnosis was confirmed in each case by skin biopsies, according to the pathologic criteria of Slavin and Woodruff.17 Of the 34 patients, 10 had no evidence of acute or chronic GVHD. Three patients without acute GVHD developed chronic GVHD following viral infection late in the course. Acute GVHD was documented in...
21 patients, this complication resolved in 9, contributed to mortality in 5, and progressed to a chronic form in 7. Patients with GVHD in whom the severity was grade 2-4 were treated initially with prednisone (2 mg/kg/day). Severe GVHD progressing despite 2-4 days of prednisone treatment was concurrently treated with intravenous infusions of horse antithymocyte globulin, (supplied by R. Condie, Ph.D., University of Minnesota). Criteria for the diagnosis of chronic GVHD included development of sclerodermatous changes and/or lichenoid lesions of the skin,16 coupled with xerostomia, xerophthalmia, or persistence of GVHD-induced abnormalities of enteric and/or hepatic function beyond 100 days posttransplant.

Isolation of Lymphocytes

Peripheral blood mononuclear cells from patients were isolated by Ficoll-Hypaque density gradient centripugulation. Cells forming rosettes with neuraminidase-treated sheep red blood cells (SRBC) were separated by sedimentation on secondary Ficoll-Hypaque gradients as previously described.17 When blood lymphocyte counts were too low to purify sufficient numbers of SRBC rosetting cells, analysis was performed with the total mononuclear cell fraction.

Monoclonal Antibodies Used to Define T-Cell Surface Markers

A series of monoclonal antibodies to T-cell differentiation antigens have been developed in this laboratory.11 Details regarding their preparation have been reported.9 In this study, four antibodies termed anti-Leu-1, anti-Leu-2a, anti-Leu-3a, and anti-Leu-4 were used. Anti-Leu-1 and anti-Leu-4 react with two distinct antigens present on the same population (~90%) of SRBC-rosetting lymphocytes from healthy controls; anti-Leu-2a reacts with a surface marker, termed Leu-2, which is present on a smaller subpopulation (25%-45%) of T lymphocytes. Anti-Leu-3a defines a surface marker, termed Leu-3, which is present on 47%-75% of SRBC-rosetting lymphocytes. In normal individuals, these subsets are nonoverlapping and complementary and together comprise the population of Leu-1+4+ lymphocytes. In normal individuals, the anti-Leu-2a antibody has been found to react with the same T-cell subset previously characterized by a rabbit anti-T-lymphocyte serum termed anti-TH2 (TH2+ subset), whereas anti-Leu-3a reacts with the complementary TH1- subset.5

Enumeration of Lymphocyte Subsets Using Monoclonal Antibodies

The percentage of cells binding specific mouse monoclonal antibodies was determined by indirect immunofluorescence analysis on a cytofluorograph (Ortho Instruments, Westwood, Mass., model FC200-4800 A 50) or a Fluorescence Activated Cell Sorter IV (Becton-Dickinson Co., Mountainview, Calif.). Cells were incubated with the appropriate antibody preparation or its control, developed with fluorescein-conjugated goat anti-mouse antibody and examined for fluorescence relative to that of appropriate negative controls as previously described.10

Results of phenotype analyses of patients were compared with results from normal controls and examined for statistically significant differences using the Student’s t test. Differences with p < 0.05 were considered significant.

RESULTS

In these studies the peripheral blood lymphocytes of 34 marrow transplant recipients were examined, of whom 23 were tested repeatedly following transplantation. Three stages of graft development were studied: days 1–30 as an index of early lymphoid development; days 30–100 as the period in which patients develop and usually resolve acute GVHD, and >100 days as the time in which durable graft-host tolerance or chronic GVHD is evident.

When peripheral blood sheep red blood cell rosetting lymphocytes from 12 normal individuals were stained with these antibodies, 80% (±8%) reacted with anti-Leu-1 and 80% (±9%) reacted with anti-Leu-4. Of these Leu-1+4+ lymphocytes, 64% (±24%) were stained by anti-Leu-3a and 36.7% (±12%) reacted with anti-Leu-2a. The ratio of Leu-2/Leu-3 T cells in this control group was 0.64 ± 0.3 and was relatively constant over time. For example, in multiple sequential testings of one individual, the percentage of cells reacting with anti-Leu-2a and anti-Leu-3a varied within 8% and 11% of the mean, respectively.

In Table 1, we have summarized results of sequential T-lymphocyte subset quantitations performed on patients at different stages of the posttransplant course. As can be seen, significant deficiencies of Leu-2+ cells were observed only in the early stages of engraftment (<1 mo), and only in the patients who did not develop GVHD. The number of circulating Leu-3+ T cells was reduced in all patients during the first month after grafting. Significant deficiencies of Leu-3+ (helper) T cells were regularly observed in patients with GVHD, both in the acute and chronic phases of disease. In contrast, patients who did not develop GVHD, or experienced resolution of this process, developed Leu-3+ T-cell numbers that approached those of controls.

As shown in Table 1, lymphocyte counts of the individual marrow transplant recipients studied exhibited considerable variability, dependent on the stage of hematopoietic recovery at which they were studied and independent of the status of graft-host tolerance. To better analyze the course of reconstitution of the two major T cells subsets in these patients as a group, we

<p>| Table 1. Quantitative Measurements of Lymphocyte Populations Bearing the Leu-2 and Leu-3 Determinants |
|-----------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Normals</th>
<th>&lt;1 mo GVHD</th>
<th>1-3 mo GVHD</th>
<th>&gt;3 mo GVHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-2+ lymphocytes/cumm</td>
<td>534 ± 308</td>
<td>268 ± 56</td>
<td>903 ± 939</td>
<td>580 ± 227</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Leu-3+ lymphocytes/cumm</td>
<td>923 ± 241</td>
<td>194 ± 107</td>
<td>248 ± 136</td>
<td>750 ± 343</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
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therefore determined the proportional representation of these cells as defined by a ratio of the absolute number of Leu-2 and Leu-3 cells/10^9 lymphocytes analyzed.

As shown in Fig. 1, there was a striking increase in the proportion of Leu-2/Leu-3 T cells in nearly all patients studied during the first month posttransplant, whether or not the patients developed acute GVHD. Thus, the ratio of Leu-2/Leu-3 T cells in 5 patients without GVHD was 3.6 ± 2.0 (p < 0.01) and in 7 patients with grades 1–2 GVHD was 3.5 ± 2.0 (p < 0.001). Of 6 patients with severe acute GVHD (grades 3–4), the Leu-2/Leu-3 ratio was normal in 2 and elevated in 4.

Figure 2 shows the results of cytofluorographic analyses measuring the proportional representation and fluorescence intensity of Leu-2 and Leu-3 cells in a normal individual (A), and in a representative patient (B), with grade 2 acute GVHD. The greater fluorescence intensity as well as the greater number of Leu-2+ cells from the patients compared to those from the normal donor can be readily seen. As in this case, highly intense staining with anti-Leu-2a was a characteristic feature of Leu-2+ cells when the ratio of Leu-2/Leu-3 T cells was elevated. It should also be noted that, as in normal lymphocytes, the Leu-2 and Leu-3 antigens exhibited little or no overlap in their distribution on T cells from these patients. Thus, in 70 separate tests of the cells of 34 patients, the percentage of lymphocytes detected by a combination of anti-Leu-2a and anti-Leu-3a differed by only 4.4% from the number of Leu-4+ cells, and by 6.3% from the sum of the 2 fractions defined separately by anti-Leu-2a and by anti-Leu-3a.

Figure 2C also shows that cells from the same patient that were treated with a mixture of anti-Leu-1 and anti-Leu-4 are distributed in a highly fluorescent peak containing the same number of cells detected by either antibody alone. Although the intensity of fluorescence of T cells stained with anti-Leu-1 was generally less in patients with elevated Leu-2/Leu-3 ratios, there was concordant expression of Leu-1 and Leu-4 by T cells in this group.

After the first month, T lymphocytes from patients without GVHD exhibited proportions of Leu-2 and Leu-3 T cells that were nearly normal (Fig. 1). In contrast, the proportion of Leu-2 T lymphocytes was still markedly increased in patients with acute GVHD irrespective of grade (differences significant at a p < 0.0025 and p < 0.01 for grades 1–2 and grades 3–4, respectively). This increase in the proportion of the Leu-2 T cells principally reflected an absolute deficiency in the number of Leu-3 (helper) cells, which was detected in each patient with acute GVHD (Table 1).

Five patients with acute GVHD were studied before
A slight reduction in the proportion of Leu-2 T lymphocytes was observed in 4 of 5 cases following initiation of treatment; however, the elevated ratio of Leu-2/Leu-3 cells persisted in all but one case. In patients who experienced resolution of acute GVHD, this ratio returned to normal, due to a marked increase in the absolute number of Leu-3 cells and not to a reduction in the number of Leu-2 cells. In patients with chronic GVHD, the Leu-2/Leu-3 ratio was also elevated \( (p < 0.001) \), although not to the degree observed in patients with acute GVHD (Fig. 1).

Sequential analyses of the absolute numbers of Leu-2 and Leu-3 T cells in a patient that incurred both acute and chronic GVHD are illustrated in Fig. 3. This patient, who was transplanted for acute myelogenous leukemia (AML) in relapse, developed acute GVHD with skin rash and hepatitis on day 10 after transplantation. When first studied on day 24, the patient’s Leu-2 T lymphocytes were present in high numbers (1340/cu mm), representing 93% of the circulating T cells. Leu-3 T cells were markedly decreased in number (86/cu mm), accounting for only 6% of T lymphocytes. Treatment with prednisone (2 mg/kg/day) (Fig. 2) was followed by improvement in skin and hepatic manifestations. The absolute T lymphocyte count decreased with resolution of the hepatitis, and the proportion and absolute number of Leu-3 T lymphocytes increased slightly, but remained subnormal. Four months after transplantation, she experienced an exacerbation of hepatitis and developed a scaling erythroderma and xerophthalmia, consistent with the diagnosis of chronic GVHD. Although the absolute number of cells in both subsets had increased, there remained a marked preponderance of Leu-2+ cells. The administration of azathioprine and prednisone resulted in an improvement but not a resolution of the clinical symptoms. The balance of Leu-2/Leu-3 T cells has continued to be abnormal and is attributable to a persistent deficiency of Leu-3 cells.

In the course of these studies, we also examined whether or not the Leu-2 and Leu-3 subpopulations developing in the period of lymphoid reconstitution are the same as those detected by monoclonal antibodies that also define suppressor (OKT8+ and helper (OKT4+) T lymphocytes. In multiple tests of the cells from all groups of patients, mixtures of OKT8 and anti-Leu-2a or of OKT4 and anti-Leu-3a detected the same percentages of T lymphocytes as were defined by each of the paired antibodies tested alone, indicating that identical subpopulations of cells were defined by each antibody set (Table 2).

**DISCUSSION**

Using monoclonal antibodies directed against surface membrane components that are uniquely expressed by one of two major human T-lymphocyte subpopulations, namely Leu-2 on suppressor/killer and Leu-3 on helper/inducer T lymphocytes (elsewhere termed OKT8+ and OKT4+, respectively), we have demonstrated significant imbalances in the representation of these subsets in the peripheral blood of patients who had received bone marrow grafts. Whether or not these patients subsequently developed GVHD, the early period of lymphocyte reconstitution was marked by a relative predominance of T lymphocytes with the suppressor/killer phenotype (Leu-
and a proportional reduction of T cells with the helper/inducer phenotype (Leu-2–3+). However, those patients who did not develop GVHD, or who responded well to therapy for acute GVHD, ultimately developed a nearly normal proportion of these subsets during a 3-mo follow-up. In contrast, those patients who had symptoms of either acute or chronic GVHD almost always had an elevated ratio of Leu-2/Leu-3 T cells during the active phase of their disease. In acute GVHD, this principally reflected a deficiency in the absolute number of Leu-3 T cells, whereas in chronic GVHD, the number of Leu-2 T cells was often disproportionately increased.

Reinherz et al.15 used an absorbed equine antithymocyte globulin that was specific for suppressor/cytotoxic (formerly TH2+) T cells to examine the representation of this subset and helper/inducer (TH1–) T cells in the peripheral blood of marrow transplant recipients. Of 6 patients with chronic GVHD, they found a predominance of TH1+ cells in 4 and a complete absence of this subset in 2. Of 3 patients studied with acute GVHD, all lacked detectable TH1+ cells in their peripheral blood. These findings led the authors to postulate that acute GVHD might, in part, be a clinical manifestation of a suppressor T-cell deficiency.

This view is not substantiated by our studies. Suppressor/cytotoxic T cells predominated in all but two of the patients with acute GVHD and were numerically deficient in none. However, T cells having the Leu-2+3– phenotype generally constituted the majority of circulating SRBC-rosetting lymphocytes in the early postengraftment period, regardless of the clinical picture. This would seem to exclude the possibility that the differences between our results and those of Reinherz et al. were determined by disparate rates of lymphoid reconstitution in these two patient groups. It is also difficult to reconcile these results with a number of experimental differences that might create significant but not absolute disparities in the number of suppressor/cytotoxic T cells detected by the equine anti-TH1 and by anti-Leu-2a in GVHD (viz., the use of the receptor for SRBC as opposed to the use of the Leu-1 and Leu-4 antigens as markers for all T cells). Therefore, the failure of the equine serum to stain any SRBC-rosetting cells in 2 of 6 patients with chronic GVHD and patients with acute GVHD would suggest that this reagent defined a membrane determinant that is distinct from the Leu-2a site and may be absent or masked on activated T cells under certain conditions. This possibility is also suggested by the fact that, on the average, anti-Leu-2a and the original rabbit anti-TH1 serum react with a much larger number of SRBC-rosetting cells (~35%)21,11 than the equine anti-TH1 (~20%).13

In keeping with prior findings, preliminary evidence indicates that GVHD is associated with the appearance of Ia antigens on circulating Leu-2+ T cells,11,19 suggesting that these cells are activated by alloantigen or by signals from alloactivated Leu-3 T cells5,6 (data not shown). In regard to the mechanisms by which alloreactivity is initiated or mediated by either subset in vivo, recent studies suggest that the Leu-2 and Leu-3 molecules are involved in the recognition and response to alloantigens in vitro. Anti-Leu-2a and anti-Leu-3a block proliferation by T cells of the appropriate subset responding in mixed leukocyte culture (MLC),20 and anti-Leu-2a also blocks specific killing of allogeneic targets by MLC-primed T cells.11 It is thus of interest that the surface density of the Leu-2 molecule may be increased on circulating Leu-2+ cells in marrow transplant recipients when the ratio of Leu-2/Leu-3 T cells is elevated, as suggested by the marked increases in the fluorescence intensity of these cells stained with anti-Leu-2a.

The possibility that suppressor/cytotoxic and helper T cells recognize antigen via different surface molecules (e.g., Leu-2 and Leu-3) may be relevant to studies in animal systems that indicate that cells belonging to these two subsets recognize foreign antigens in association with different MHC-encoded molecules on accessory or target cells.21,22 Moreover, the

<p>| Table 2. Percent Distribution of T-Cell Phenotype |
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<table>
<thead>
<tr>
<th></th>
<th>Leu-2</th>
<th>OKT8</th>
<th>Leu-3</th>
<th>OKT4</th>
<th>Combined Leu-2+ OKT8</th>
<th>Combined Leu-3+ OKT4</th>
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<tr>
<td>Normals</td>
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<tr>
<td>No GVHD</td>
<td>3</td>
<td>59</td>
<td>36</td>
<td>37</td>
<td>45</td>
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</tr>
<tr>
<td>Acute GVHD</td>
<td>5</td>
<td>86</td>
<td>7</td>
<td>8</td>
<td>86</td>
<td>86</td>
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<tr>
<td>Chronic GVHD</td>
<td>7</td>
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<td>9</td>
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<tr>
<td>Late reconstitution</td>
<td></td>
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same MHC products that influence antigen recognition also determine alloreactivity by these subsets. Thus, murine T cells having the suppressor/cytotoxic phenotype respond to alloantigens controlled by MHC (mouse H-2) K- and D-regions, whereas helper T cells respond to alloantigens encoded by the H-2 I region. A similar dichotomy of T-cell responses to MHC antigens in man is suggested by the finding that Leu-3 cells recognize and respond to alloantigens encoded by the human I region counterpart, HLA-D, whereas Leu-2 T cells do not. The mechanisms leading to GVHD and the associated imbalance of Leu-2 and Leu-3 T cells in this disease must therefore be understood in terms of the unique cognitive functions of these subsets as well as their specific regulatory activities.

ACKNOWLEDGMENT

We are grateful to Cindy Stutzer, R.N., and to the nurses and staff of the Marrow Transplantation Unit, and the Attending Physicians—Robert Dinsmore, Neena Kapoor, Dahlia Kirkpatrick, Hal D. Teitelbaum and Subhash Gulati—for their valued assistance in the conduct of these studies.

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