Analysis of T Lymphocytes After Bone Marrow Transplantation Using Monoclonal Antibodies

By Robert Fox, Robert McMillan, Wayne Spruce, Patti Tani, and Donna Mason

Using monoclonal antibodies to cell surface antigens and fluorescent cell sorter analysis, we studied peripheral blood lymphocyte subsets after bone marrow transplantation (BMT). In 13 patients studied 3 mo or more after BMT, the ratio of T-cell subsets defined by antibodies OKT4 and OKT8 was reversed (OKT4/OKT8 = 0.7 ± 0.3) in comparison to normal volunteers or bone marrow donors (ratio OKT4/OKT8 = 1.7 ± 0.4) (p < 0.001). This reversed ratio persisted for up to 3 yr after BMT. In contrast to a previous report, the presence of an abnormal ratio of T-cell subsets did not correlate with clinically significant graft-versus-host disease (GVHD). In agreement with a previous study, we found increased Ia-positive T cells in BMT patients (26% ± 8%; <4% in normals (p < 0.001) and antibody OKT10 reactive cells (39% ± 20% versus 10% ± 4%) (p < 0.01), suggesting in vivo activation. However, their PBL did not react with antibody B3/25 (antitransferrin receptor), a marker found on normal PBL after in vitro activation by mitogens (BMT patients <5%; normal PBL T cells plus PHA 45% ± 11%). These results demonstrate that BMT patients have: (A) an abnormal ratio of T-cell subsets in the presence or absence of clinically significant GVHD disease so that these measurements were not useful in monitoring patients; (B) an increased number of T cells with cell surface phenotype (OKT8+, Ia+, OKT10+, B3/25-) that is distinct from normals but similar to patients with infectious mononucleosis or acquired hypogammaglobulinemia.

Bone marrow transplantation (BMT) is frequently complicated by graft-versus-host disease (GVHD). The ability to predict GVHD disease could lead to early therapeutic steps to decrease its morbidity. Using heteroantiserum TH2 that detects a subset of T cells, Reinherz et al. reported that BMT patients with GVHD had a markedly increased proportion of TH2 T cells, while BMT patients free of this complication were similar to normal donors. Recently, monoclonal antibody OKT8 (that detects the same lymphocyte subset as antisemur TH2) and antibody OKT4 (that detects a reciprocal T-cell subset) have become available. To determine if these antibodies would also be useful in monitoring BMT patients, we performed immunofluorescent staining and cytofluorographic analysis on peripheral blood lymphocytes from 13 patients, including 10 who were free of any clinical evidence of GVHD. In contrast to the previous study, we found that the proportion of T-cell subsets was significantly different from normal in virtually all BMT patients. Therefore we could not use these measurements to identify patients with clinically significant GVHD. We did, however, find an increased frequency of activation antigens (Ia, OKT10, and a newly developed monoclonal T305) on T cells in the majority of BMT patients, thus confirming and extending the previous reports.

MATERIALS AND METHODS

Patient Population

We studied 13 patients who had undergone BMT: 9 with acute leukemia, 3 with aplastic anemia, and 1 with acute myelofibrosis (Table I). Each patient received bone marrow from an HLA and mixed lymphocyte culture (MLC) matched sibling. Recipient conditioning and pre- and posttransplantation care have been previously described in detail. Briefly, patients with acute leukemia and acute myelofibrosis were prepared for transplantation with cycosine arabinoside (5 mg/kg) on days (–)18 and (–)3; cyclophosphamide (80–100 mg/kg) on day (–)15, and total body irradiation (1000 rad) on day (–)1. Patients with aplastic anemia were prepared with cyclophosphamide (50 mg/kg) on days (–)5, 4, 3, and 2. All patients received methotrexate, 15 mg/sq m on day (+)1 and 10 mg/sq m on days (+)3, 6, 11 and weekly thereafter for 100 days, for prevention of GVHD. Occasional doses were omitted for clinical reasons. All patients received corticosteroids (0.5 mg/kg) beginning on day 21 and continuing for at least 3 mo in tapering doses. In patients with active acute or chronic GVHD, higher doses of corticosteroids were used.

Patients 1–4 were studied early, during acute GVHD, and again later after all clinical manifestations had cleared; patients 5, 7, and 8 were studied during persistent GVHD (patient 5 developed symptoms of acute GVHD on day 21, which persisted until her death on day 68; patient 7 developed acute GVHD that progressed to a pattern consistent with chronic GVHD; and patient 8 had no evidence of acute GVHD but on day 248 manifested typical skin findings of chronic GVHD that persisted); patients 9–13 were sampled several months after transplantation at a time when no clinically apparent GVHD was present. Acute and chronic GVHD...
were diagnosed, and the severity was staged using previously reported criteria. In each of the BMT patients having acute GVHD that we studied, the clinical symptoms of GVHD occurred before or simultaneously with the reappearance of circulating lymphocytes. Thus, it was not possible to study these BMT patients' peripheral blood lymphocytes (PBL) prior to development of A-GVHD symptoms.

Bone marrow donors and age- and sex-matched normal subjects served as controls. Patients with primary Sjögren's syndrome (10 patients with no other associated connective tissue disease), scleroderma (3 patients), or acquired immunodeficiency (4 patients) were seen at Scripps Clinic. These patients had received no (corticosteroids or immunosuppressive) medications for at least 3 mo prior to study. In view of the increased frequency of herpes virus infections in BMT patients, we also studied 2 cases of herpes zoster and 3 cases of lymphocytoma occurring in otherwise healthy young adults.

**Antibodies and Fluorescence-Activated Cell Sorter Analysis**

Total T cells, defined by the presence of the receptor for sheep erythrocytes, were determined by antibody OKT11 that detects this receptor, while mature T cells (representing about 90% of total T cells in normal volunteers) were detected by antibody OKT3. Antibody OKT4 and OKT8 were used to evaluate T-cell subsets, containing inducer/helper and suppressor/cytotoxic activities, respectively. In both normals and BMT patients, T-cell subsets expressed either OKT4 or OKT8 (but not both) based on two-color immunofluorescent staining patterns employing Biotin-Leu 3a and fluorescein-conjugated Leu 2a (that detect the same subsets as OKT4 and OKT8, respectively) and rhodamine-Avidin (Vector, San Dalton antigen present on activated PBL and acute lymphocytic leukemia cells. Two-color immunofluorescent staining to detect Ia-positive T cells and T-cell subsets was performed using biotin-conjugated monoclonal antibody and rhodamine-conjugated avidin followed by fluorescent-conjugated anti-Ia antibody (FL-SG2). Murine myeloma proteins MOPC 21 and GPC 7, having no known antireceptor activity, were used as negative control to determine nonspecific binding of murine IgG1 and IgG2 immunoglobulins, respectively.

Peripheral blood lymphocytes (PBL) were prepared from heparinized blood by centrifugation over a Ficoll-Hypaque gradient following monocyte depletion using carbonyl iron. The use of carbonyl iron depletion did not alter the ratio of T-cell subsets, but significantly reduced the nonspecific antibody staining attributable to Fe receptors on these cells. After washing with 5% fetal calf serum, the PBL were treated with monoclonal antibody and fluorescent F(ab')2 anti-mouse Ig as previously described. The number of stained cells was determined by fluorescence-activated cell sorter analysis (FACS II, Becton-Dickinson, Mountain View, Calif.). For two-color immunofluorescent analysis, cytosegment preparations of stained cells were examined under a fluorescent microscope. (Carl Zeiss, Inc., New York, N.Y.) equipped with vertical illumination and excitation barrier filter combinations for fluorescein 450 nm to 490 nm excitation and rhodamine 540 nm to 550 nm excitation.

**RESULTS**

**Abnormal Ratio of T-Cell Subsets in Patients After Bone Marrow Transplantation**

Table 2 shows that the cell surface characteristics of PBL in normal volunteers were similar to the bone marrow donor's in terms of mature T cells (defined by antibody OKT3), as well as similar proportions of OKT4 ' and OKT8 ' cells with an OKT4/OKT8 ratio of 1.6–1.7. In contrast, all BMT patients studied more
than 3 mo after transplantation showed a significant reversal of this ratio of T-cell subsets (OKT4/OKT8 = 0.7 ± 0.3) \( (p < 0.001 \text{ by Student's } t \text{ test}) \). In three patients (nos. 5, 7, 8), clinically significant GVHD was present at the time of the evaluation. However, their T-cell patterns were not different \( (p > 0.1) \) from the other 10 BMT patients who did not have clinically significant GVHD.

Next, the BMT patients' PBL were further characterized with additional monoclonal antibodies (Table 3). Antibody OKT11 (that detects the sheep cell receptor on T cells)\(^6\) reacted with 82% ± 5% of the cells, in close agreement with the antibody OKT3 results noted above. B cells (8% ± 2%) and OKM-1 reactive cells (16% ± 7%) were also present. No significant staining was noted with antibody OKT6 that detects an antigen on immature T cells.\(^6\)

### Table 2. Abnormal Ratio of T-Cell Subsets After Bone Marrow Transplantation (% Cells Reactive)

<table>
<thead>
<tr>
<th>Source of PBL</th>
<th>Prednisone</th>
<th>Lymphs (per cu mm)</th>
<th>OKT3* (T Cells)</th>
<th>OKT4*</th>
<th>OKT8*</th>
<th>Ratio (OKT4/OKT8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (12)</td>
<td>—</td>
<td>2.125 ± 395</td>
<td>75 ± 3</td>
<td>49 ± 4</td>
<td>29 ± 3</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>BMT donor (4)</td>
<td>—</td>
<td>2.060 ± 425</td>
<td>79 ± 3</td>
<td>51 ± 3</td>
<td>32 ± 3</td>
<td>1.6 ± 0.3</td>
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<tr>
<td>BMT patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>147</td>
<td>1,470</td>
<td>90</td>
<td>36</td>
<td>50</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>660</td>
<td>59</td>
<td>35</td>
<td>28</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
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<td>590</td>
<td>60</td>
<td>14</td>
<td>35</td>
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<tr>
<td>4</td>
<td>97</td>
<td>760</td>
<td>60</td>
<td>27</td>
<td>37</td>
<td>0.6</td>
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<tr>
<td>5</td>
<td>68</td>
<td>164</td>
<td>72</td>
<td>30</td>
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<tr>
<td>6</td>
<td>115</td>
<td>2,072</td>
<td>81</td>
<td>24</td>
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<td>7</td>
<td>397</td>
<td>810</td>
<td>77</td>
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<td>8</td>
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<td>77</td>
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<td>9</td>
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<td>30</td>
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<tr>
<td>10</td>
<td>292</td>
<td>1,800</td>
<td>73</td>
<td>30</td>
<td>43</td>
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</tr>
<tr>
<td>11</td>
<td>448</td>
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<tr>
<td>12</td>
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<tr>
<td>13</td>
<td>1,120</td>
<td>2,280</td>
<td>67</td>
<td>30</td>
<td>35</td>
<td>0.9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1,445 ± 744</td>
<td>70 ± 10</td>
<td>28 ± 6</td>
<td>40 ± 8</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>

\*BMT days after transplantation when analysis of PBL was performed.

**Lymphocyte Cell Surface Antigens Occurring in Association With Acute Graft-Versus-Host Disease**

In four patients it was possible to obtain PBL coincident with the onset of acute GVHD (28 ± 2 days after transplant). The median lymphocyte count was 684/cu mm. The distribution of surface antigens was: OKT3* (63% ± 12%), OKT4* (37% ± 14%), and OKT8* (21% ± 6%). None of these patients subsequently developed chronic GVHD. As noted above, in each case the OKT4/OKT8 ratio had reversed by 3 mo after transplantation. Unfortunately, none of our patients had sufficient PBL prior to onset symptoms of acute GVHD to allow immunofluorescent analysis.
Comparison to PBL From Patients With Infectious Mononucleosis, Acquired Hypogammaglobulinemia, and Sjogren’s Syndrome

The reversed ratio of T-cell subsets noted in BMT patients was also seen in patients with acute infectious mononucleosis, who also showed a markedly increased frequency of certain activation antigens (Ia, OKT10, T305) but not B3/25 (Table 4). In contrast, two otherwise healthy patients with herpes zoster had PBL with a normal ratio of lymphocyte subsets and no significant increase in activation antigens (p > 0.1). Since clinical features in some patients with GVHD are similar to those of patients with Sjogren’s syndrome or scleroderma, we studied patients with these diseases. We found their lymphocyte patterns to be significantly different from the BMT patients (Table 4) in terms of T-cell subsets and presence of activation antigens (p < 0.001). Thus, the PBL from BMT patients have a phenotype that is similar to that of certain viral infections but distinct from other inflammatory autoimmune diseases.

To determine if the observed alteration in BMT patients was due to corticosteroids, we studied 5 patients with rheumatoid arthritis (prednisone 5-10 mg/day) and 3 patients with polymyositis (prednisone 30-60 mg/day). We found the distribution of their T-cell subsets to be similar to normal (OKT4/OKT8 = 1.5-2.5). Thus, corticosteroids did not appear to directly affect the T-cell subset proportions.

DISCUSSION

An initial step in understanding the immunologic events associated with bone marrow transplantation is the precise identification of lymphocyte subsets that reconstitute these patients. In using monoclonal antibodies to characterize lymphocyte subsets, we noted that PBL from our BMT patients differed significantly from those of normal volunteers in terms of T-cell subsets and presence of activation antigens. These differences occurred in BMT patients with and without symptoms of GVHD. These results differ from those of Reinherz et al. who noted that BMT patients had an abnormal ratio of T-cell subsets only when they had GVHD. Because our patients' cells were not tested with antiserum TH2, we cannot exclude the possibility that they were OKT8-positive, TH-2-negative. However, we conclude that monoclonal antibodies to T-cell subsets were not useful in identifying patients with clinically significant GVHD. We did note that BMT patients with GVHD had a slightly higher incidence of Ia-positive T cells than BMT patients free of this complication (36% versus 20%), but additional patients with GVHD must be analyzed before the merit of this measurement can be evaluated in the diagnosis of GVHD.

Our results are in agreement with de Bruin et al., who also noted a low OKT4/OKT8 ratio in the majority of their BMT patients. In expanding these previous observations, we also studied 4 BMT patients at an earlier time after transplantation (less than 1 mo and at the time of acute GVHD symptoms) and found that these patients had relatively normal ratios of T-cell subsets (average of OKT4/OKT8 = 1.8) but that the ratio in each patient reversed by 3 mo posttransplant. De Bruin et al. also noted an increase in OKT10-positive cells and suggested that this was due to their relative immaturity. Subsequent studies have demonstrated that OKT10 can also be induced by in vitro activation and our finding of other activation antigens (Ia- and T305-positive T cells) would tend to support this hypothesis. The observation that BMT express only some activation antigens (i.e., lack of transferrin receptor) emphasizes the difference between in vivo activation (in BMT, infectious mononucleosis, and acquired hypogammaglobulinemia patients) and in vitro mitogen-activated cells. These differences may reflect the nature of the in vivo stimulus or the particular subsets responding.

The functional significance of the OKT4+ cells early after transplantation in some patients remains unknown, but these lymphocytes may be helper cells that initiate a response against alloantigens (or viral antigens) present in the recipient. The significance of the
OKT8+ cells in these BMT patients also remains unclear, but they may be suppressor cells responsible for maintaining graft tolerance20 similar to that noted in animal models.21 Our results are consistent with this observation, since lymphocytes with this phenotype (OKT8+, Ia-, OKT10+) in infectious mononucleosis patients22,23 have been shown to possess suppressor cell function. An altered ratio of T-cell subsets may also reflect occult infection by viruses such as EBstein-Barr virus or cytomegalovirus.21-24 It is less likely that medications such as prednisone are directly responsible, since patients with rheumatoid arthritis or polymyositis had a normal ratio despite similar doses of steroid; however, such medicines may be indirectly responsible by allowing occult viral infections to occur. Patients with chronic inflammatory diseases such as primary Sjogren's syndrome or scleroderma, which share certain clinical features with GVHD patients,18,24 also differ from BMT patients in their ratio of T-cell subsets and absence of activation antigens.

These studies identify the types of T cells that repopulate patients after bone marrow transplantation. Ultimately, a more thorough understanding of the functional and phenotypic characteristics of these cells may be translatable into rational therapeutic maneuvers in the management of these complicated patients.

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REFERENCES

7. Trowbridge I, Omary B: Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Proc Natl Acad Sci USA 78:3039, 1981
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R Fox, R McMillan, W Spruce, P Tani and D Mason