T-Cell Subpopulations Identified by Monoclonal Antibodies After Human Marrow Transplantation. I. Helper-Inducer and Cytotoxic-Suppressor Subsets

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Peripheral blood helper-inducer and cytotoxic-suppressor T-cell subpopulations in patients receiving marrow transplants for the treatment of acute leukemia or severe aplastic anemia were quantitated on the fluorescence-activated cell sorter (FACS) using the monoclonal antibodies OKT4 and OKT8, respectively. The relative (percent) and absolute number of OKT4+ cells were severely and persistently depleted for up to 2.7 yr posttransplant. In contrast, the percent and absolute number of OKT8+ cells began to recover within the first 60 days of transplant and subsequently remained at normal or high levels for periods of up to 7.3 yr. There was no significant difference in percent or absolute numbers of OKT8+ cells for patients with or without acute graft-versus-host disease (GVHD). The reversal of the normal OKT4:OKT8 ratio (2:1) occurred regardless of whether the recipient was given an allogeneic, syngeneic, or autologous transplant and regardless of whether or not acute or chronic GVHD developed. The reversed ratio was due in the first 3 mo posttransplant to low numbers of OKT4+ cells and later to a combination of low numbers of OKT4+ and high numbers of OKT8+ cells. Normalization and then an increase in the number of OKT8+ cells correlated with increasing time posttransplant and not with resolution of acute GVHD.

MATERIALS AND METHODS

Normal adult values for the E+, OKT4+, and OKT8+ populations were established at the Fred Hutchinson Cancer Research Center using healthy volunteer blood donors. Cord blood was obtained from healthy normal-term infants delivered at Swedish Hospital, Seattle. Wash. All patients in the study underwent bone marrow transplantation for acute lymphoblastic leukemia, acute nonlymphoblastic leukemia, or severe aplastic anemia at the Fred Hutchinson Cancer Research Center, Swedish Hospital, or the University of Washington Hospital, Seattle. Peripheral blood lymphocytes of 41 patients were studied for the purposes of characterizing T-cell profiles during the first 3 mo posttransplant and those of a further 30 patients from 3 mo to 7 yr posttransplant. Patients receiving allogeneic grafts were given either intermittent methotrexate (MTX) or cyclosporin-A (CyA) as prophylaxis for GVHD. The syngeneic and autologous recipients were given no postgrafting immunosuppression. Patients were grouped into the following categories:

1. Patients studied during the first 100 days following marrow transplantation.
   (A) Recipients of syngeneic or autologous marrow grafts.
   (B) Recipients of marrow grafts from an HLA genotypically identical sibling.
      (i) on prophylactic methotrexate with no evidence of GVHD occurring during the first 100 days.
      (ii) on prophylactic methotrexate with grade II-IV acute GVHD occurring during the first 100 days.
      (iii) on prophylactic cyclosporin-A with grade II-IV acute GVHD occurring during the first 100 days.
   (iv) on prophylactic cyclosporin-A with grade II-IV acute GVHD occurring during the first 100 days.
2. Patients studied more than 100 days after receiving marrow grafts from an HLA genotypically identical sibling.
   (A) Patients with no evidence of GVHD.
   (B) Patients with chronic GVHD.

Bone Marrow Transplantation

Details on the selection of patients and donors for transplantation, on the conditioning regimens for transplantation, and on the transplant procedure have been described. The clinical and histopathologic staging systems for both acute and chronic GVHD have been described, as have the therapy of these complications with corticosteroids, antithymocyte globulin, and other cytotoxic
agents.\textsuperscript{1-12} Cyclosporin-A was administered daily to patients in groups (iii) and (iv) starting on the day before marrow infusion at a dose of 12.5 mg/kg/day in 2 divided doses and tapering by 5%/wk from day 50 on. Evidence for allogeneic marrow engraftment was obtained by monitoring the peripheral blood count, determination of marrow cellularity, and frequent monitoring of blood genetic markers. Moderately severe to severe GVHD (grades II–IV) was diagnosed according to previously published criteria.\textsuperscript{7,4} Patients were treated with prednisone.\textsuperscript{11} Twenty-three percent of the test samples in patients with acute GVHD were obtained before therapy, and 77% were obtained after prednisone was started. Since results of the T-cell subset analyses did not differ significantly between treated and untreated patients with acute GVHD, they were combined for presentation. Similarly, there were no significant differences between results in patients with active or resolved acute GVHD.

**Absolute Lymphocyte Count**

Absolute lymphocyte counts were derived from the total white blood cell count and differential. Using standard laboratory techniques, these were performed on all blood samples tested in this study.

**Preparation of Peripheral Blood Mononuclear Cells (PBMC)**

Heparinized blood was diluted 1:1 with phosphate-buffered saline (PBS), and mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Lymphocyte Separation Medium, Biozymics, Kensington, Md.). Cells were washed 3 times in PBS, counted, and resuspended at $3 \times 10^7$/ml in RPMI 1640 (Gibco, Grand Island, N.Y.) and 20% fetal calf serum (FCS). The cell suspensions were allowed to sit overnight at room temperature. The following day, cells were assayed for percent spontaneous rosette formation and stained by indirect immunofluorescence with monoclonal antibody.

**Indirect Immunofluorescence Assay**

Twenty-five microliters of monoclonal antibody were added to washed cells and incubated at 4°C for 30 min. After washing and centrifuging through a cushion of FCS, 200 μl of fluoresceinated goat anti-mouse IgG (Meloy Laboratories, Springfield, VA) was added, and the cells were incubated at 4°C for 30 min, washed again, and then resuspended in 1 ml of cold PBS with 1% bovine serum albumin and 0.1% sodium azide. The cell suspension was filtered through nitex screening fabric of 41-μ-mesh size (Tetko, Inc., Elmsford, N.Y.) prior to analysis on the Fluorescence Activated Cell Separator II (FACS II) (Becton-Dickinson, Mountain View, Calif.). Monoclonal antibodies used in this study included OKT4\textsuperscript{4,14} and OKT8.\textsuperscript{14} Each of these antibodies, which are of the IgG subclass, was used at a dilution of 1:600. Two murine monoclonal IgG antibodies specific for mouse leukemia virus protein (clones 9E8 and 16C1) were used as negative controls for the indirect immunofluorescence assay.\textsuperscript{13}

**Assay for Spontaneous E-Rosette Formation**

One million peripheral blood mononuclear cells in 0.33 ml were mixed with 0.01 ml of AET-treated sheep red blood cells (at concentrations of 10% packed cell volume, approximately $3 \times 10^7$/ml). The mixture was centrifuged at 150 g for 10 min and then incubated on ice for 1 hr. The pellet was then gently resuspended and rosettes counted, using a 1% crystal violet solution. Mononuclear cells with 3 or more attached sheep red blood cells were counted as rosettes and scored as E+. The absolute number of E+ cells was derived from the absolute lymphocyte count.

Quantitation of Lymphocyte Subpopulations on the FACS II

Indirect immunofluorescent staining for monoclonal antibodies OKT4 and OKT8 was analyzed with a FACS II. The percent of lymphocytes stained with antibody OKT4 or OKT8 was determined after correcting for cells stained with the negative control antibody (9E8 or 16C1). The absolute number of OKT4-positive (OKT4+) and OKT8-positive (OKT8+) lymphocytes was derived from the absolute lymphocyte count. The percent of E+, OKT4−, and OKT8− cells was derived from the percent of OKT4+ and OKT8+ lymphocytes after adjustment for percent of E+ cells. The absolute numbers of E+, OKT4−, and OKT8− cells were derived from the absolute E+ count after adjusting the percent OKT4+ and OKT8+ lymphocytes for the percent E+ cells. For each experiment, a blood sample from at least one normal individual was included.

**RESULTS**

**Normal Healthy Adults**

Twenty-seven laboratory personnel or normal bone marrow donors were tested on 53 different occasions. For individuals tested more than once, only the mean value of all their tests was entered into the calculation of the average for normals (Table I). The average relative number of E+ lymphocytes in peripheral blood of healthy adults was 82% ± 10%. The absolute number of E+ cells was 1812 ± 959/mm. The average relative number of OKT4+ and OKT8+ lymphocytes was 40% ± 9% and 19% ± 7%, respectively, and the OKT4:OKT8 ratio was 2:1:1. The absolute number of OKT4+ and OKT8+ cells was 884 ± 517 and 447 ± 312, respectively. All values given represent the mean ± 1 SD. To assess variation, two normal individuals were each tested on 9 and 6 occasions during the time of the study (Table I). The relative number of E+, OKT4−, and OKT8− cells was found to be relatively stable. A greater variation was seen for absolute cell numbers.

**Cord Blood**

Cord blood samples from eight healthy normal-term infants were found to give an equivalent proportional number of E+ cells, but approximately double the absolute number found in healthy adults (Table 1). The relative number of OKT4+ and OKT8+ cells in cord blood was 12% ± 10% and 4% ± 6%, which is significantly less than found in adults. The absolute number of OKT4+ and OKT8+ cells, however, was less than found in adults. The number of OKT4− and OKT8− cells was markedly increased. The OKT4:OKT8 ratio for cord blood cells was 3.0.

**Patients**

**E+ cells.** Syngeneic and autologous recipients had normal relative numbers but low absolute numbers of E+ cells during this period. All allogeneic recipients
tested during the first 100 days posttransplant had low relative and absolute numbers of E+ cells regardless of the presence or absence of acute GVHD and of whether MTX or CyA was used as immunosuppression (Table 2). Patients with chronic GVHD had low relative and absolute numbers of E+ cells between 3 and 6 mo posttransplant, but after this time, long-term survivors both with and without chronic GVHD had normal relative and absolute numbers of E+ cells.

**OKT4+ cells.** Marrow transplant recipients had persistently low relative and absolute numbers of OKT4+ cells regardless of whether they received allogeneic, syngeneic, or autologous grafts, regardless of the underlying disease for which they were transplanted (e.g., leukemia versus aplastic anemia), regardless of the conditioning regimen, and regardless of whether or not they developed acute or chronic GVHD (Table 2, and Figs. 1 and 2). The deficiency of OKT4+ cells persisted for up to 2.7 yr posttransplant.

**OKT8+ cells.** Marrow transplant recipients of allogeneic grafts tended to have low relative and absolute numbers of OKT8+ cells during the first 30 days (Table 2, and Figs. 3 and 4). The relative numbers of OKT8+ cells increased thereafter, so that by day 90 the relative number of OKT8+ cells was significantly higher than normal. The presence or absence of chronic GVHD did not affect the relative or absolute numbers of OKT8+ cells.

### Table 1. Absolute (per cu mm) and Relative Numbers of E+, OKT4+, and OKT8+ Peripheral Blood Lymphocytes in Normal Adults* and Normal-Term Infants†

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>E+ Cells</th>
<th>OKT4+ Cells</th>
<th>OKT8+ Cells</th>
<th>E+, OKT4+ and OKT8+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent</td>
<td>Absolute No.</td>
<td>Percent</td>
<td>Absolute No.</td>
</tr>
<tr>
<td>Normal adults (27)†</td>
<td>82 ± 10</td>
<td>1,812 ± 959</td>
<td>40 ± 9</td>
<td>884 ± 517</td>
</tr>
<tr>
<td>Normal-term infants (8)†</td>
<td>78 ± 11</td>
<td>3,912 ± 901</td>
<td>12 ± 10</td>
<td>623 ± 524</td>
</tr>
<tr>
<td>Normal donors tested repeatedly over a 6-mo period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor 1</td>
<td>11 samples</td>
<td>85 ± 7</td>
<td>2,933 ± 1,042</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>Donor 2</td>
<td>11 samples</td>
<td>87 ± 6</td>
<td>1,517 ± 392</td>
<td>45 ± 4</td>
</tr>
</tbody>
</table>

*Hoffman et al.† reported a mean value for 17 normal adults: OKT4 = 46 ± 8 and OKT8 = 32 ± 8 without correction for false positive staining.† All results expressed as mean ± 1 SD.† Number in parenthesis indicates the number of individuals tested.

### Table 2. Absolute and Relative Numbers of E+, OKT4+, and OKT8+ Peripheral Blood Lymphocytes in Patients After Bone Marrow Transplantation

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Number of Individuals</th>
<th>Number of Tests</th>
<th>E+ Cells</th>
<th>OKT4+ Cells</th>
<th>OKT8+ Cells</th>
<th>E+, OKT4+, and OKT8+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent</td>
<td>Absolute No.</td>
<td>Percent</td>
<td>Absolute No.</td>
<td>Percent</td>
<td>Absolute No.</td>
</tr>
<tr>
<td>Syngeneic recipients</td>
<td>4</td>
<td>8</td>
<td>81 ± 5†</td>
<td>825 ± 637</td>
<td>29 ± 14</td>
<td>225 ± 205</td>
</tr>
<tr>
<td>AutoLOGOUS recipients:</td>
<td>1</td>
<td>4</td>
<td>79 ± 7</td>
<td>1,358 ± 182</td>
<td>13 ± 2</td>
<td>228 ± 28</td>
</tr>
<tr>
<td>Allogeneic recipients:</td>
<td>7</td>
<td>19</td>
<td>70 ± 14</td>
<td>744 ± 385</td>
<td>16 ± 3</td>
<td>154 ± 87</td>
</tr>
<tr>
<td>Allogeneic recipients: MTX, no acute GVHD</td>
<td>18</td>
<td>46</td>
<td>69 ± 14</td>
<td>890 ± 562</td>
<td>15 ± 13</td>
<td>155 ± 131</td>
</tr>
<tr>
<td>Allogeneic recipients: CyA, no acute GVHD</td>
<td>7</td>
<td>20</td>
<td>76 ± 8</td>
<td>843 ± 384</td>
<td>22 ± 7</td>
<td>227 ± 107</td>
</tr>
<tr>
<td>Chronic GVHD, Day 100–180</td>
<td>7</td>
<td>11</td>
<td>70 ± 14</td>
<td>1,191 ± 1,131</td>
<td>12 ± 7</td>
<td>180 ± 167</td>
</tr>
<tr>
<td>Chronic GVHD, Day 181–266</td>
<td>12</td>
<td>12</td>
<td>75 ± 13</td>
<td>1,423 ± 1,166</td>
<td>19 ± 9</td>
<td>329 ± 234</td>
</tr>
<tr>
<td>Healthy allogeneic long-term survivors (no chronic GVHD), Day 270–2990</td>
<td>11</td>
<td>11</td>
<td>75 ± 16</td>
<td>1,820 ± 1,083</td>
<td>20 ± 8</td>
<td>495 ± 382</td>
</tr>
</tbody>
</table>

*MTX, methotrexate; CyA, cyclosporin-A.† All results expressed as mean ± SD.‡ Bolded figures represent significant differences from range of p < 0.05 to p < 0.0005.
T CELL SUBSETS AFTER MARROW GRAFTING

Fig. 1. Relative number of OKT4+ cells in the blood of patients after marrow transplantation. MTX, methotrexate; CyA, cyclosporin-A; N, number of patients in each group.

absence of GVHD (acute or chronic) had no apparent effect on the number of OKT8+ cells observed, either early (up to day 98) or late (up to day 2660) after transplantation. The relative numbers of OKT8+ cells was also found to be elevated in patients receiving autografts on a graft from a syngeneic donor (Table 2 and Fig. 3).

OKT4:OKT8 ratios. All patient categories showed reversal of the normal 2:1 OKT4:OKT8 ratio (calculated from data in Table 2). Early after transplantation, this was due to low numbers of OKT4+ cells and later was accentuated both by low numbers of OKT4+ cells and by high numbers of OKT8+ cells. The percent OKT4+ and OKT8+ cells and the OKT4:OKT8 ratios, determined serially in four patients during the posttransplant period, are illustrated in Fig. 5. Patients UPN 1273 (on prophylactic cyclosporin-A) and UPN 1214 (on prophylactic methotrexate) had no evidence of GVHD. The differences seen between these two patients were not charac-

Fig. 2. Absolute number per cu mm of OKT4+ cells in the blood of patients after marrow transplantation. N, number of patients in each group.

Fig. 3. Relative number of OKT8+ cells in the blood of patients after marrow transplantation. N, number of patients in each group.

Fig. 4. Absolute number of OKT8+ cells in the blood of patients after marrow transplantation. N, number of patients in each group. (-----)Mean value for allogeneic recipients with GVHD. (-----)Mean value for allogeneic recipients without GVHD. (2)Range (mean ± 1 SD) of values for normal individuals.
Figure 5. Serial determination of percent OKT4+ and OKT8+ cells in the blood of four patients after marrow transplantation. Patient UPN 1273 received cyclosporin-A for GVHD prophylaxis, and patients UPN 1214, 1237, and 1239 received methotrexate. The normal values for percent OKT4+ (40% ± 9%) and OKT8+ (19% ± 7%) are indicated by bar graphs. "+" indicates onset of acute GVHD. "o" indicates treatment with prednisone. (__)Range (mean ± 1 SD) of OKT4+/OKT8+ ratios for normal individuals.

During the first 3 mo posttransplant, syngeneic recipients had low relative and absolute numbers of E+, OKT4−, and OKT8− cells. Allogeneic recipients without acute GVHD had normal relative but low absolute values, while allogeneic recipients with acute GVHD had high relative and normal absolute numbers. While relative numbers became normal beyond 3 mo posttransplant in allogeneic recipients, absolute numbers remained low.

**DISCUSSION**

The similarity of OKT4+ and OKT8+ cell profiles in the different patient groups posttransplant was striking. Neither the type of transplant, the primary disease of the patient, the immunosuppressive regimen, nor the presence or absence of GVHD appeared to influence the rate or extent of reconstitution of cells expressing the T4 and T8 antigens. The OKT4+ subset, defined as the helper-inducer T-cell subset, remained persistently depleted long-term posttransplant. This is surprising in view of the fact that while helper T-cell function in an in vitro immunoglobulin biosynthesis assay is impaired in all transplant recipients in the first 100 days and in long-term survivors with chronic GVHD, it is often normal in long-term survivors without chronic GVHD. In vivo antibody production in response to the T-dependent antigens keyhole limpet hemocyanin and bacteriophage QX174 follow similar patterns and cannot be distinguished from normal in healthy marrow graft recipients living longer than 1 yr. In contrast to the persistently low OKT4+ cell numbers, numbers of OKT8+ cells were initially normal and later high. Again, this pattern occurred in all patient categories. However, when patients' cells are examined in functional assays, greater heterogeneity of activity of the cytotoxic-suppressor subset is seen. Cytotoxic T cells are present...
in acute and chronic GVHD in humans (Tsoi, unpublished observations); nonspecific OKT8+ suppressor cells are found only in patients with chronic GVHD, and specific suppressor cells in long-term survivors without chronic GVHD. It would appear unlikely that these findings are simply due to a state of immunologic immaturity, since none of the normal 9-mo term infants had a reversed OKT4:OKT8 ratio, while all marrow transplant recipients did, including those surviving 9 mo and longer.

The results in the 71 patients of the present study are different from the findings of Reinherz et al., who identified T-cell subpopulations in blood of 16 marrow transplant recipients using the TH2 antisera, which is known to identify the suppressor–cytotoxic T-cell subset and is the equivalent of the OKT5 monoclonal antibody. In our studies we have used the OKT8 monoclonal antibody, which includes all OKT5-positive cells as well as identifying an additional 5%–10% of suppressor–cytotoxic cells. Reinherz et al. found no TH2+ cells (OKT5-positive cells, which are included in the OKT8+ population) in patients with acute GVHD until shortly before cessation of disease activity when TH2+ cells reappeared. They found no TH2+ cells in two of six patients with chronic GVHD and normal T-cell profiles in patients without GVHD. In contrast, we found no distinct change in the helper versus suppressor–cytotoxic subsets in patients with acute or chronic GVHD. The explanation for these differences is not clear. However, as demonstrated in Figs. 1–4, the levels of OKT8+ cells (suppressor–cytotoxic) in patients with acute and chronic GVHD vary considerably. Thus, results reported may vary considerably from one study to another if the numbers of patients are relatively small.

In summary, helper–inducer T cells, as measured by monoclonal antibody OKT4, were found to be severely and persistently low in autologous, syngeneic, and allogeneic marrow transplantation patients for up to 2.7 yr postgrafting. T cells of suppressor–cytotoxic phenotype, as measured by monoclonal antibody OKT8, however, began to appear in increasing relative and absolute numbers within 3 mo of transplant and subsequently remained at high levels for periods of up to 7.3 yr. Thus, a reversal was seen in the normal OKT4:OKT8 ratio in all patient groups studied. No significant change was seen in the relative or absolute numbers of OKT8+ cells in patients with either acute or chronic GVHD.

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