Peripheral Blood Lymphocyte Subsets after Allogeneic Bone Marrow Transplantation: Reconstitution and Correlation with the Occurrence of Acute Graft-Versus-Host Disease

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Bone marrow transplantation (BMT) is now an established procedure to treat aplastic anaemia, leukaemia, severe combined immunodeficiency and other malignant tumours and genetic disorders. It is still associated with high morbidity and mortality, mainly due to opportunistic infections and graft-versus-host disease (GVHD). Monitoring immunological function of BMT recipients provides the opportunity of investigating the pathophysiology of immunodeficiency, opportunistic infections, graft rejection and GVHD. An initial step to understand the immunologic events following BMT is the study of lymphocyte subsets in peripheral blood of these patients.¹-⁵ The aims of the present study are (1) to follow the reconstitution of peripheral blood lymphocyte subsets in Chinese patients receiving allogeneic BMT and (2) to see whether the occurrence of acute GVHD influences this reconstitution pattern.

MATERIALS AND METHODS

I. Patient population

We studied 21 patients who received HLA-identical sibling BMT at the BMT Centre of Queen Mary Hospital, University of Hong Kong. These patients included 12 with acute myeloblastic leukaemia (AML), 3 with acute lymphoblastic leukaemia (ALL), 4 with chronic myeloid leukaemia (CML), 1 with multiple myeloma (MM) and 1 with severe aplastic anaemia (SAA).

II. Pre-transplantation conditioning

Patients with AML or CML were conditioned with either cyclophosphamide (CY) 60 mg/kg for 2 days plus 12 Gy total body irradiation (TBI) in 6 fractions over 3 days or busulphan 4 mg/kg for 4 days plus CY 60 mg/kg for 2 days. The same doses of CY plus TBI were given to ALL patients. The MM patient was conditioned with melphalan (110 mg/m² for 1 day) plus TBI (12 Gy in 6 fractions over 3 days) and the SAA patient received CY (60 mg/kg for 2 days) plus total lymphoid irradiation (6 Gy in 6 divided doses over 2 days).

SUMMARY Peripheral blood lymphocyte subsets were enumerated at regular intervals during the first year after allogeneic bone marrow transplantation (BMT) in 21 Chinese patients. Eight of these patients had acute graft-versus-host disease (GVHD) while they were assessed at the time of engraftment. Our results show in patients receiving allogeneic BMT: (1) T and NK cells were the predominant lymphocyte subsets in the early reconstitution stage while B cells were severely depleted; (2) absolute numbers of the major lymphocyte subsets normalised in 4–5 months; (3) an increased percentage of T cells that expressed the activation antigen HLA-DR and a reversed CD4:CD8 ratio were observed throughout the first 12 months after BMT; (4) patients with acute GVHD had significantly higher white cell count and NK cell percentage than those not complicated by acute GVHD.
III. GVHD prophylaxis

All patients received GVHD prophylaxis consisting of methotrexate (MTX) and cyclosporin A (CsA). MTX 15 mg/m² was given intravenously on day 1 and then 10 mg/m² on days 3, 6 and 11. CsA was started on day -1 at a dose of 1.5 mg/kg intravenously 12 hourly. Subsequent dosage was adjusted according to whole blood CsA level which was maintained between 200 ng/ml and 300 ng/ml. Oral CsA was commenced at 5 mg/kg BD when mucositis subsided and patients could feed orally. CsA dose was reduced by 5% each week from day 50 and discontinued at day 180.

IV. GVHD

Of the 21 patients, 8 patients developed grade II-IV acute GVHD: 6 with grade II, 1 with grade III and 1 with grade IV. In 5 patients there was histological evidence of acute GVHD on skin biopsy. The remaining 3 patients had skin rash and/or diarrhoea clinically compatible with acute GVHD and subsequently responded to treatment with methylprednisolone. Four patients developed chronic GVHD: 3 evolved from acute GVHD, 1 de novo.

V. Study protocol

Peripheral blood lymphocyte subsets were assessed by flow cytometry within 6 days after engraftment (defined as white cell count >0.5 × 10⁹/l) and then at 1.5, 3, 6, 9, and 12 months after engraftment. By the end of the study period, 5 patients were dead and 2 had relapses of their diseases; hence only 14 patients were able to complete all the assessments.

VI. Antibodies and flow cytometry analysis

All antibodies were obtained from Becton Dickinson (Mountain View, CA 94039). Leu-4 (CD3) and Leu-12 (CD19) were used to detect total T and B cells, respectively. T-helper/inducer cells were marked by Leu-3a (CD4) and Leu-2a (CD8) was used to detect T-suppressor/cytotoxic cells and some NK cells. Natural killer cells were defined as cells with the phenotype CD16 (Leu-11c) and/or CD56 (Leu-19) but CD3⁻. Standard dual colour flow cytometry using whole blood was employed as previously described. Briefly, 100 µl of peripheral blood anticoagulated with EDTA were mixed with 10 µl of the antibodies directly conjugated with fluorochromes. After incubation for 30 minutes, the cells were washed twice with phosphate-buffered saline. Red cells were lysed and white cells were fixed using the Whole Blood Lysing Reagent Kit of Coulter (Hialeah, FL 33014-0486). Stained cells were analysed by the Coulter Profile II flow cytometer. Total white cell count and differential counts were determined by the Technicon II counter.

VII. Statistical analysis and normal values

Comparison of numerical values between groups was performed by the Wilcoxon Rank Sum test. Normal ranges of lymphocyte subsets were previously established on a group of 129 healthy volunteers.

RESULTS

I. Recovery of white blood cells and differential counts

Total white cell and lymphocyte counts returned to the lower limit of normal range by approximately the sixth month after engraftment. Normalisation of neutrophil count occurred at about the second month and the number of monocytes was normal at the time of engraftment (Fig. 1).

II. Recovery of lymphocyte subsets

Fig. 2 shows the mean percentages of lymphocyte subsets after BMT. At the time of engraftment, B cells were virtually non-existent in peripheral blood. The B cell percentage increased to the lower normal limit approximately 3 months later. Percentages of T and NK cells were within the normal range throughout the first year after transplantation although a downward trend was seen in both subsets.

Fig. 3 shows the mean absolute numbers of lymphocyte subsets after BMT. Total T lymphocyte number was depressed early after transplantation and returned to normal at about 4-5 months. During the first month, CD4⁺ and CD8⁺ cells were in comparable numbers. Subsequently CD4⁺ cells recovered at a slower rate than CD8⁺ cells, resulting in a CD4/CD8 ratio of about 0.5 during the first 12 months following BMT (Fig. 4). The number of B cells in blood became normal within 4-5 months post-transplantation. The number of NK cells was on the lower side of the normal range throughout the first year.

The activation marker HLA–DR was detected on 40–50% of T cells (25–35% of total lymphocytes) during the first year after transplantation. Although there was a downward trend, the percentage at the end of the first year was still significantly higher than the corresponding mean values in normal individuals (10.4% of T cells or 7.3% of all lymphocytes were HLA–DR⁺, p < 0.01).

III. Lymphocyte subsets in association with acute GVHD

Eight of the patients were complicated by acute GVHD grade II or above during the first assessment. They had significantly higher total white cell count and NK cell percentage than those without acute GVHD. The DR⁺ T cell absolute number was also higher in the group with acute GVHD, but the difference was not statistically significant (Table 1).
Fig. 1. Mean total white cell and differential counts per μl. Horizontal bars mark the values of mean ± 1 standard deviation. Upper and lower horizontal lines represent the upper and lower normal limits, respectively. X-axis: weeks after engraftment.
Fig. 2. Mean percentages of lymphocyte subsets vs weeks after engraftment. Horizontal bars mark the values of mean + 1 standard deviation. Upper and lower horizontal lines represent the upper and lower normal limits, respectively.
Fig. 3. Mean absolute numbers of lymphocyte subsets per μL. Horizontal bars mark the values of mean ± 1 standard deviation. Upper and lower horizontal lines represent the upper and lower normal limits, respectively. x-axis: weeks after engraftment.
DISCUSSION

Using dual colour flow cytometry to characterise lymphocyte subsets, we studied the pattern of immune reconstitution in 21 patients receiving allogeneic BMT. Early after BMT, T and NK cells were the major subsets of lymphocytes but B cells were virtually non-existent. CD4+ and CD8+ cells were in approximately comparable numbers in the first few weeks but CD8+ cells subsequently outnumbered CD4+ cells by about 2-fold. The absolute numbers of both T and B cells normalised 4-5 months post-BMT whereas the absolute number of NK cells reached the lower normal limit soon after engraftment.

It has been shown by the combined analysis of cell surface and sex chromosome marker that recipient T cells (mainly CD4+) persist during the first few weeks post-grafting.\(^8,9\) In our study the CD4:CD8 ratio was initially above 1.0 but subsequently decreased to approximately 0.5. These findings are consistent with the theory that a small number of recipient CD4+ cells remain after conditioning but donor CD8+ cells repopulate at a faster rate than CD4+ cells.\(^5,9-12\) Whether the increase is due to CD8 bright T cells or CD8\(^{dim}\) NK cells or both is not answered in the present study. Other studies had identified NK cells that account for the elevated levels of circulating CD8+ cells post-BMT,\(^1,12,13\) but we have not found a greatly increased NK cell absolute number or percentage. Low CD4:CD8 ratios may be related to acute GVHD and viral infections, but such correlations remain an area of contention.\(^14\) The reversed CD4:CD8 ratio persisted for up to 1 year after BMT in our study and other reports showed this continued to be so 2-3 years after BMT.\(^2,3\)

Several reports have found an increased percentage of NK cells after BMT.\(^3,15,16\) In our study the percentage of NK cells was initially at the upper part of the local reference range (25-35%) and this decreased to the lower part (10-15%) by the end of the first year. The difference may be explained by difference in selection of patients, since this and other studies have found a significantly higher NK percentage in those patients with acute GVHD. Alternatively, we have reported a higher local reference range

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**Table 1.** Comparison of peripheral blood lymphocyte subsets in patients with and without acute GVHD at the time of engraftment.

<table>
<thead>
<tr>
<th>Subset</th>
<th>aGCHD(^*) (n=8)</th>
<th>without aGCHD (n=13)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. absolute number (per μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White cell</td>
<td>5,406 ±3,839* *</td>
<td>1,541 ±780</td>
<td>0.0049</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>490 ±292</td>
<td>320 ±194</td>
<td>0.2119</td>
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<tr>
<td>CD19</td>
<td>6.1 ±3.7</td>
<td>7.4 ±14.2</td>
<td>0.2345</td>
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<tr>
<td>CD3</td>
<td>302.4 ±148.2</td>
<td>220.2 ±133.4</td>
<td>0.3142</td>
</tr>
<tr>
<td>CD4</td>
<td>156.0 ±97.8</td>
<td>110.9 ±86.2</td>
<td>0.5252</td>
</tr>
<tr>
<td>CD8</td>
<td>217.0 ±155.3</td>
<td>140.4 ±105.6</td>
<td>0.2382</td>
</tr>
<tr>
<td>NK</td>
<td>182.4 ±156.0</td>
<td>83.8 ±71.4</td>
<td>0.1140</td>
</tr>
<tr>
<td>DR(^+) T</td>
<td>213.3 ±75.7</td>
<td>115.3 ±104.8</td>
<td>0.0757</td>
</tr>
<tr>
<td>b. CD4:CD8</td>
<td>0.94 ±0.69</td>
<td>1.16 ±20.67</td>
<td>0.4822</td>
</tr>
<tr>
<td>c. percentage</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lymphocyte</td>
<td>14 ±11</td>
<td>22 ±14</td>
<td>0.0558</td>
</tr>
<tr>
<td>CD19</td>
<td>1.3 ±0.5</td>
<td>1.2 ±0.6</td>
<td>0.3906</td>
</tr>
<tr>
<td>CD3</td>
<td>64.4 ±13.7</td>
<td>72.2 ±13.0</td>
<td>0.2290</td>
</tr>
<tr>
<td>CD4</td>
<td>34.7 ±18.7</td>
<td>40.9 ±15.6</td>
<td>0.4864</td>
</tr>
<tr>
<td>CD8</td>
<td>42.6 ±8.7</td>
<td>40.7 ±11.1</td>
<td>0.6642</td>
</tr>
<tr>
<td>NK</td>
<td>35.0 ±9.0</td>
<td>22.7 ±13.3</td>
<td>0.0315</td>
</tr>
<tr>
<td>DR(^+) T</td>
<td>36.7 ±8.7</td>
<td>31.5 ±16.7</td>
<td>0.7181</td>
</tr>
</tbody>
</table>

* aGCHD = Acute graft-versus-host disease.
** mean ±SD
of NK cell percentage in comparison to that of the Western populations and this may have obscured the increase of NK cell percentage in BMT patients.

The increased proportion of T cells that express HLA-DR in the first year after BMT was also reported by others. The increased number of activated T cells could be a result of in vivo activation with alloantigens. Alternatively, DR-bearing T cells may reflect an intermediate stage in differentiation between thymocytes and mature T cells. The presence of an immature population of T cells is supported by studies which detected an abnormally high proportion of CD38+ T cells in peripheral blood lymphocytes.

A large body of evidence has concluded that mature donor T lymphocytes are essential for the recognition of antigenic disparity and subsequent initiation of acute GVHD. Recent data suggested that NK cells may also be important in the pathogenesis of acute GVHD. Comparing patients with and without acute GVHD, we have found the former group had a significantly higher percentage of NK cells. The white cell count was also significantly increased but this was likely to be due to the use of corticosteroid to treat acute GVHD. It was not possible in our study to measure the lymphocyte subsets before the onset of acute GVHD since all these patients developed this complication before the first blood samples were taken. Hence it is not known whether these markers could be used to predict GVHD leading to early therapeutic steps to decrease morbidity and mortality. In one report, a drop in CD4:CD8 ratio preceded the development of severe GVHD in most patients. Other studies have found a marked increase in CD8+ cells or a decrease in CD4:CD8 ratio during acute GVHD. These features were not seen in the present nor in other reports. It seems that abnormalities in the CD4:CD8 ratio did not correlate with the occurrence of GVHD.

REFERENCES


