T Lymphocyte Repopulation and Differentiation After Bone Marrow Transplantation. Early Shifts in the Ratio Between T4⁺ and T8⁺ T Lymphocytes Correlate With the Occurrence of Acute Graft-Versus-Host Disease

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Acute graft-versus-host disease (GVHD), a major complication of allogeneic bone marrow transplantation (BMT), is probably mediated by T lymphocytes present in the marrow graft. In this study, the repopulation of the peripheral blood with T4⁺ and T8⁺ T cells was investigated during the period preceding the occurrence of acute GVHD. Twenty-four allogeneic and 11 autologous BMT recipients were monitored from day 4 post-BMT onward by the use of monoclonal antibodies, indirect immunofluorescence, and flow cytometry. The recipients of allogeneic transplants received methotrexate as GVHD prophylaxis. Similar recovery patterns for T4⁺ and T8⁺ T cells were found following autologous and allogeneic BMT. However, lymphoid repopulation occurred at a clearly faster rate after autologous BMT. T4⁺ T cells were the first to reappear in the peripheral blood, followed by T8⁺ T cells 4–7 days later. The T8⁺ T cell reconstitution occurred at an even faster rate in patients who were to develop grade II–IV GVHD, as compared with those with grade 0–I GVHD, thus leading to an earlier decrease in the T4/T8 ratio. Of 10 patients with a T4/T8 ratio <2.5 at day 19, 9 developed grade II–IV GVHD and 1 showed no GVHD. Of 14 patients with a ratio >2.5 at that time, only 2 developed grade II–IV and 12 grade 0–I GVHD (p < 0.001). In the 11 patients developing grade II–IV GVHD, the T4/T8 ratio decreased to values <2.5 before the first clinical symptoms of GVHD in 9; it coincided in one and occurred later in another patient. Thus, early monitoring of the T4/T8 ratio can distinguish patients at risk of developing grade II–IV GVHD.

ALLOGENEIC BONE MARROW transplantation (BMT) is being increasingly applied as a treatment modality for acute leukemia,¹ chronic myelogenous leukemia,²,³ and severe aplastic anemia,⁴ as clinical experience has shown that a permanent cure in these conditions can be achieved with BMT. Following transplantation of marrow from an HLA-identical sibling donor, about 60% of the recipients will develop acute graft-versus-host disease (GVHD).⁵⁻⁷ T lymphocytes in the marrow graft are likely to mediate this reaction.⁸ Our preliminary studies of T cell phenotypes during the period preceding the clinical symptoms of GVHD seemed valuable for its prediction and may be helpful in enabling early therapeutic intervention, with probably a greater chance of success.⁹ Here, we extend our data and compare our results after allogeneic BMT with those obtained after autologous BMT.

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For this purpose, marrow graft recipients were monitored twice weekly between 4 and 40 days post-BMT. T cell subsets bearing the T4 and T8 markers were enumerated using monoclonal antibodies (MCA), indirect immunofluorescence, and analysis by flow cytometry. To investigate the influence of persisting recipient cells on the ratio between T4⁺ and T8⁺ T cells, four sex-mismatched BMT recipients were studied using quinacrine staining of Y bodies in combination with T cell typing for T4 and T8.

MATERIALS AND METHODS

Patients

The clinical data on the 35 patients studied are listed in Table 1. Twenty-four patients received an allogeneic marrow graft from an HLA-identical, MLC-nonreactive sibling, and 11 received autologous marrow. The 22 patients with acute leukemia in remission, the 3 patients with chronic myelogenous leukemia in first chronic phase, and 4 patients with non-Hodgkin’s lymphoma were prepared with cyclophosphamide (60 mg/kg/day x 2) and 800 rad total body irradiation. Two patients with severe aplastic anemia (nos. 5 and 10) were prepared with cyclophosphamide (50 mg/kg/day x 4) and fractionated total lymphoid irradiation (200 rad x 10). The third patient (no. 11) received a single dose of 750 rad instead of fractionated irradiation. One patient with testicular teratocarcinoma (no. 27) received cyclophosphamide (60 mg/kg/day x 4) and VP16-213 (300 mg/sq m x 4); another (no. 29) received iophosphamide (60 mg/kg/day x 5) and VP16-213 (200 mg/sq m x 3). The patient with Ewing’s sarcoma (no. 32) was prepared with a single dose of melphalan (4.5 mg/kg). Twenty-two of the 24 patients undergoing allogeneic BMT received a median dose of 1.9 x 10⁸/kg nucleated bone marrow cells (range 1.6–2.8 x 10⁸/kg). Two patients (nos. 16 and 19) received bone marrow depleted of lymphocytes by counterflow centrifugation;¹⁰ they received 0.18 and 0.45 x 10⁸ cells/kg, respectively. For autologous grafting, at least 2 x 10⁸ cells/kg were provided.
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CFU-GM was always greater than 80%. All GVHD ing, recovery of cells/kg were cryopreserved using standard techniques; after thaw-mg/day for 14 days; 3 of these received it as the initial therapy for

in first chronic phase; NHL, non-Hodgkin's lymphoma; TCT, testicular teratocarcinoma; EWS, Ewing sarcoma.

GVHD prophylaxis.5.

clinical criteria'2 and was always confirmed histologically.'3 Six Heparinized venous blood samples were obtained twice a week

syndrome; Sep. septicemia.

section).

recipients of allogeneic marrow were administered methotrexate (20 mg/kg/ I kg/day) and 6 with high-dose methylprednisolone

OKT3.PAN (Ortho Pharmaceutical Co., Raritan, NJ) at a dose of 5 elsewhere.'4

for 2 days). Eight patients with GVHD were treated with prednisone (1-2 mg/ kg/day) and 6 with high-dose methylprednisolone (20 mg/kg/12 hr for 2 days). Eight patients received monoclonal antibody OKT3.PAN (Ortho Pharmaceutical Co., Raritan, NJ) at a dose of 5 mg/day for 14 days; 3 of these received it as the initial therapy for GVHD.

Analysis of T Cell Subpopulations

Heparinized venous blood samples were obtained twice a week from day 4 post-BMT till day 40 and processed within 12 hr. Before engraftment, at least 40–50 ml blood was necessary for each monitoring event. Enrichment for T cells was done as described elsewhere.16 The following monoclonal antibodies (MCA) were used:
OKT4,\textsuperscript{13} OKT8,\textsuperscript{14} and the OKT8-like MCA FK18.\textsuperscript{17} The OKT antibodies were purchased from Ortho Diagnostics; FK18 was kindly supplied by Dr. F. Koning (Leiden, The Netherlands). The freeze-dried MCA of the OKT series were reconstituted with 1 ml phosphate-buffered saline (PBS) per vial; 40 \( \mu \)l of a 1:40 dilution of reconstituted MCA was added to 0.25-0.50 \( \times 10^6 \) cells. The same number of cells was incubated with 40 \( \mu \)l of a 1:500 dilution of ascitic fluid containing FK18. As the second antibody, goat anti-mouse Ig, conjugated with fluorescein isothiocyanate (GAM/Ig/FITC), was used (Nordic Immunological Laboratories, Tilburg, The Netherlands). For flow cytometry, 40 \( \mu \)l of a 1:50 dilution was added. The indirect immunofluorescence assay and the analysis by flow cytometry were performed as described elsewhere.\textsuperscript{14}

**Fluorescence Technique for the Demonstration of Y-Chromatin in Interphase Nuclei**

Ficoll-Isopaque separated mononuclear peripheral blood cells were washed with PBS supplemented with 1% bovine serum albumin (PBS/BSA) and vitally stained with either OKT4 or FK18 MCA for 30 min at 4°C. After washing with PBS/BSA, the T\( ^+ \) and T\( ^- \) T cells were incubated for 30 min at 4°C with goat anti-mouse Ig, conjugated with tetramethylrhodamine isothiocyanate (GAM/Ig/ TRITC; Nordic Immunological Laboratories) at a 1:40 final dilution. The cell suspensions were washed with PBS/BSA, and cytospin slides were made using 50 \( \mu \)l of the cell suspensions adjusted to 3–5 \( \times 10^6 \) nucleated cells per slide. Slides were air-dried and fixed in ethanol/ether (3/1 v/v) at room temperature for 8 min. They were then immersed in a 0.75% solution of quinacrine dihydrochloride (Sigma, St. Louis, MO) in methanol at room temperature for 15 min, rinsed 3 times in distilled water, and mounted in Aquamount (Gurr, BDH Chemicals Ltd., Poole, UK). In 200 T\( ^+ \) and T\( ^- \) T cells, single green-fluorescent nuclear bodies (Y-bodies) were enumerated, using a Zeiss standard 18 microscope equipped with the Ploem epi-illuminator IV F1 and filter combinations suitable for TRITC and FITC fluorescence.

**Statistical Analysis**

When clinical symptoms of GVHD became manifest, the results obtained afterwards were excluded from analysis because of possible influence of corticosteroid therapy\textsuperscript{18} or treatment with OKT3.PAN on the T cell subset composition. Details of the statistical analysis of the data are given in Results.

**RESULTS**

**Incidence and Severity of GVHD**

GVHD developed in 15 of the 24 recipients of an allogeneic BMT. Of these 15 patients, 4 developed grade I GVHD, 7 grade II, 3 grade III, and 1 grade IV GVHD. The median time of occurrence of symptoms was at day 27 (range 11–55 days post-BMT).

**Repopulation of the Peripheral Blood with T4\( ^+ \) and T8\( ^- \) T Cells**

To study the pattern of T\( ^+ \) and T\( ^- \) T cell repopulation following BMT, the individual data were pooled per patient group (grade 0–I GVHD and grade II–IV GVHD in allogeneic BMT recipients and autologous BMT recipients) and per time interval, as indicated in the legend to Fig. 1. Absolute numbers of lymphocytes and of T\( ^+ \) and T\( ^- \) T cells are shown in Fig. 1.

In allogeneic BMT recipients, absolute lymphocyte counts were lowest at between days 8 and 11 and then started to increase again. From day 18 onward, the absolute lymphocyte count increased clearly faster and to higher levels in patients developing grade II–IV GVHD than in patients showing grade 0–I GVHD. Nevertheless, the lymphocyte count remained below the normal range in both groups during the entire period of study. The T\( ^+ \) T cell subset started to
increase from day 11 onward, and reached a level of ±100/cu mm on the average within 2 wk. It remained at this level until day 40. T4+ T cell numbers were similar in patients with grade II–IV GVHD and with grade 0–I GVHD. The repopulation of the peripheral blood with T8+ T cells lagged behind that with T4+ T cells, but, in patients developing grade II–IV GVHD, T8+ T cells repopulated clearly faster and to much higher levels as compared with patients with grade 0–I GVHD. The absolute number of T8+ T cells, however, was within the normal range from about day 25 onward; however, this occurred only in the case of patients developing grade II–IV GVHD. The numbers remained below the normal range in patients with grade 0–I GVHD, at least until day 40.

A similar pattern of lymphoid cell repopulation, but at a much faster rate, was seen after autologous BMT. Here, the absolute lymphocyte count now started to increase from day 8 onward. The T4+ T cells were again the first to increase, followed by the T8+ T cells. The overall absolute lymphocyte count was within the normal range from about day 15 onward, in contrast to the allogeneic situation, in which the normal range was not reached within 40 days. Although T4+ T cells did not reach normal numbers during our period of observation, they were significantly higher than T4+ T cell counts in the allogeneic BMT recipients (p < 0.01 with Student’s two-tailed t test). Again, T8+ T cells reached normal levels, but much earlier (from day 15 onward) than in allogeneic BMT recipients (p < 0.01 at between days 11 and 24).

**Early Decrease in the T4/T8 Ratio**

**Prior to Acute GVHD**

When sequential determinations of T4/T8 ratios, rather than absolute numbers of T4+ and T8+ T cells, were plotted (Fig. 2), the discrimination between the groups of patients who were to develop grade II–IV GVHD and those with grade 0–I GVHD became even more apparent. The T4/T8 ratio was decreased significantly earlier in patients who were to develop grade II–IV; in these patients, the T4/T8 ratio started to decrease from day 14 onward. In patients with grade 0–I GVHD, the decrease started later and more gradually. Between days 18 and 22, the T4/T8 ratio of grade II–IV and grade 0–I GVHD patients was (mean ± standard deviation) 2.1 ± 0.9 and 4.7 ± 1.7, respectively (p < 0.001); between days 22 and 25, these were 1.3 ± 1.0 and 3.2 ± 1.2, respectively (p = 0.005; two-tailed Student’s t test) (Fig. 2).

Because the difference in T4/T8 ratios became significant for the first time between days 18 and 22, the T4/T8 ratios at this time were plotted against the grade of GVHD that ultimately developed (Fig. 3). A T4/T8-ratio of 2.5 at this time proved to discriminate optimally between the grade II–IV and grade 0–I patient groups.

Detailed data indicating how far in advance GVHD could have been predicted in the individual patients,
and the ultimate grade of GVHD they developed, are presented in Fig. 4. The length of the bars indicates, for each individual patient, the time lapse between the last monitoring event with a T4/T8 ratio >2.5 and the next event at which the T4/T8 ratio had dropped below 2.5. Thus, if more frequent monitoring would have been performed, this transgression could most likely have been picked up earlier. In 9 of 11 patients who were to develop grade II–IV GVHD, the T4/T8 ratio dropped below the 2.5 limit prior to the clinical onset of GVHD; in patient no. 10, the onset of GVHD (day 11) occurred when the T4/T8 ratio was still <2.5, and in patient no. 15, the transgression coincided with the first symptoms of GVHD. Because corticosteroid therapy was started at days 12 and 28, respectively, both patients were excluded from subsequent statistical analysis. In the remaining 9 patients, the T4/T8 ratio fell below 2.5 at a median of 16 days (range days 8–22). The 13 patients with grade 0–I GVHD, passed the 2.5 threshold after a median of 24 days post-BMT (range days 12–40) (p < 0.001 with Wilcoxon’s rank sum test).

Survival of Recipient T Cells

To investigate the influence of persisting recipient cells on the ratio between T4+ and T8+ T cells, four patients with sex-mismatched donors were studied with quinacrine staining for Y bodies in combination with T cell typing for T4 and T8 (Fig. 5). Prior to BMT, normal patterns were seen in both recipients and donors. In the two female patients (nos. 17 and 20), the low percentage of Y-body positive (Y+) T4+ and T8+ T cells at day 5 were similar to those found in normal female controls. In patient no. 17, T4+ Y+ and T8+ Y+ T cells reached the normal range for males at day 19; in patient 20, these values were still intermediate at day 36. In the two male patients (nos. 14 and 21), the percentages of T4+ Y+ T cells at day 5 were still similar to those found in normal males, whereas the T8+ Y+ percentage had decreased to intermediate values. In patient 14, the T4+ Y+ and T8+ Y+ T cells reached the donor level at day 19. Patient 21 showed an earlier replacement of the T8+ compartment by donor T8+ T cells as compared with the replacement of the T4+ compartment by donor T4+ T cells; the T8+ Y+ percentage was at the donor level at day 19, whereas this did not occur until day 43 for the T4+ Y+ percentage. Thus, patient 21 was the only one in whom the disappearance of recipient T4+ and T8+ T cells occurred at different rates; in the other three patients studied, the recipient-derived fractions of T4+ and T8+ T cells were similar, which means that recipient T cells did not contribute to alterations in the T4/T8 ratio.

Correlation Between Absolute Lymphocyte Count and T4/T8 Ratio

The increase in the absolute lymphocyte count coincided with a faster repopulation of the T8+ T cell subset as compared with that of the T4+ T cell subset, resulting in a decrease in the T4/T8 ratio. Thus, it could be expected that the increase in absolute numbers of lymphocytes for the individual patients would correlate linearly with the decrease in T4/T8 ratios. Regression lines for individual patients were plotted.
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using logarithmic scales, and are shown in Fig. 6. An inverse correlation was found in both autologous (mean ± SD, \( r = -0.63 ± 0.18 \)) and allogeneic (\( r = -0.74 ± 0.19 \)) BMT recipients. As can be seen from Fig. 6, two patients receiving allogeneic marrow (nos. 13 and 19) clearly differed from the others in that their regression lines had positive correlation coefficients (\( r = 0.89 \) and 0.72, respectively). This anomaly can be explained by the following observations. Patient 13 started out with a T4/T8 ratio of 0.8 at day 8, which increased to 3.4 at day 12 and then decreased to 2.2 at day 15. Afterwards, GVHD became manifest and corticosteroid therapy was started. Patient 19 showed a very slow lymphoid repopulation, with a gradually increasing T4/T8 ratio (from 2.9 to 6.6 between days 8 and 29). Therefore, these two patients were excluded from the calculation of the average regression lines. When average regression lines were calculated for autologous and allogeneic BMT recipients, the intercepts were similar (mean ± SD, 3.34 ± 1.65 versus 3.93 ± 1.74), but the slopes differed significantly (\(-1.33 ± 1.05 \) versus \(-10.23 ± 7.48 \), respectively; \( p = 0.001 \) with Student’s two-tailed t test). This represented the earlier inversion of the T4/T8 ratio as a function of the absolute lymphocyte count in the allogeneic BMT recipients.

DISCUSSION

The correlation between the development of GVHD and changes in T cell subpopulations preceding the onset of clinical symptoms was studied using MCA recognizing the T4 and T8 surface markers. T cell repopulation and differentiation following BMT had already been studied by several groups using anti-T4 and anti-T8 MCA.19-25 For obvious reasons, monitoring was usually started after engraftment, i.e., when the white blood cell count was >1,000/cu mm;922 only occasionally was earlier monitoring done, e.g., starting from day 14 onward.23,24 There is consensus that, as tested at >1 mo posttransplantation, the T4/T8 ratio is not influenced by the presence of acute GVHD. Hence, we anticipated that if shifts in T cell subpopulations were to precede the onset of GVHD, such changes had to take place very early after BMT. Therefore, we started T cell subset analysis as early as day 4 after BMT, i.e., 1–2 wk before engraftment.

Cell suspensions enriched for T cells by AET-rosetting were analyzed because, despite narrow-angle gating, flow cytometry of Ficoll-isolated mononuclear cells was frequently complicated by myelomonocytic cells, reticulocytes, and nucleated red cells outnumbering the cells to be studied. This enrichment procedure had been shown not to induce shifts in T4 and T8 T cell subsets.25 Although lymphocyte counts were generally low during this period after BMT (i.e., <500/cu mm), the percentages of T4⁺ and T8⁺ T cells could be determined with great accuracy as a result of the technique used (flow cytometry). The early repopulation of the peripheral blood with T4⁺ and T8⁺ T cells was, in absolute numbers, similar in recipients of autologous and allogeneic marrow. T4⁺ T cells were the first to reappear in the circulation, followed by T8⁺ T cells, but the T8⁺ T cells repopulated at a faster rate than did the T4⁺ T cells, ultimately resulting in an inverted T4/T8 ratio. We found that preceding, or sometimes simultaneously with, the development of clinical symptoms of grade II–IV GVHD, a much faster proliferation of T8⁺ T cells takes place than is observed in
patients with grade 0–I GVHD. Since the increase in absolute numbers of T4⁺ T cells is identical in both groups of patients, this explains the earlier decrease in the T4/T8 ratio found in the grade II–IV GVHD patients.

Because immunosuppressive therapy might induce shifts in T cell subsets, thereby influencing the conclusions to be drawn from the data relating to GVHD, we excluded all monitoring events from the analysis after GVHD had become clinically manifest and therapy for GVHD had been started. The significantly earlier decrease in the T4/T8 ratio in patients who were to develop grade II–IV GVHD as compared with those with grade 0–I GVHD, as found in this study, can therefore be related to GVHD. Methotrexate as a possible cause can also be excluded, as all patients transplanted with allogeneic marrow received this drug as prophylaxis for GVHD.

The faster repopulation of the peripheral blood with T cells after autologous BMT can be explained by the fact that these patients did not receive methotrexate, a myelosuppressive drug. Thus, it can be concluded from our data that an early decrease in the T4/T8 ratio correlates with the development of grade II–IV GVHD. In fact, patients with a T4/T8 ratio <2.5 at day 19 usually develop only grade I GVHD or none at all (Fig. 3). Their ratios decrease below the 2.5 level at a median of 24 days posttransplantation, i.e., 8 days later than in patients with grade II–IV GVHD (Fig. 4). The two groups ultimately show similar T4/T8 ratios (<1.0), in agreement with the published data.²⁻⁴⁻¹²⁻²⁴

The percentages of T4⁺ and T8⁺ T cells of recipient origin at day 19 did not differ in 3 of 4 patients studied (nos. 14, 17, and 20) (Fig. 5). Hence, the T4/T8 ratios determined at that time were not influenced by the presence of remaining recipient T cells. In the fourth patient (no. 21), the proportion of T cells of recipient origin was higher for T4⁺ T cells than for T8⁺ T cells. Nevertheless, this fact did not influence the predictive value of the T4/T8 ratio in relation to the ultimate GVHD status (grade 0) in this patient. However, it is striking that, in the period during which acute GVHD can develop, at least the T cell compartment of the peripheral blood is composed of a mixture of donor and recipient cells.

An inverse linear correlation was found between the increase in absolute lymphocyte numbers and the decrease in the T4/T8 ratio following BMT (Fig. 6). The slope of the average regression line is significantly steeper in the allogeneic BMT group than in the autologous BMT group, i.e., the T4/T8 ratio decreases much faster in relation to the increasing absolute lymphocyte numbers in recipients of allogeneic marrow than in recipients of autologous marrow. However, because the rate of increase in absolute lymphocyte numbers after autologous BMT is much faster and reaches higher levels than after allogeneic BMT, the decrease in the T4/T8 ratio after autologous BMT occurs at a similar rate or even faster (Fig. 2). In summary, analysis of the kinetics of T4⁺ and T8⁺ T cell repopulation after allogeneic marrow grafting with methotrexate as a prophylactic regimen for acute GVHD shows that determination of the T4/T8 ratio at day 19 can predict acute GVHD. Whether this also holds true for patients receiving cyclosporine A as prophylaxis for GVHD is presently under study.

The volumes of blood necessary for each monitoring event prevented daily monitoring in this series. However, these volumes might be reduced if flow cytometric analysis of whole blood samples were used; this would obviate the need for laborious T cell isolation as well. This way, daily monitoring of the T4/T8 ratio would become feasible, thus enhancing the predictive ability of this procedure.

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