Differential CD26-Mediated Activation of the CD3 and CD2 Pathways After CD6-Depleted Allogeneic Bone Marrow Transplantation

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Patients who have undergone allogeneic bone marrow transplantation (allo-BMT) are susceptible to a variety of opportunistic infectious complications in the months to years after engraftment. Impaired in vitro T-cell functions have been documented in these patients, and these T-cell dysfunctions contribute to the prolonged immune deficiency after allo-BMT. In the present study, we examined the expression of CD26 as well as the reconstitution of CD26-mediated T-cell costimulation via the CD3 and CD2 pathways at various times in patients aged greater than 18 years after CD6-positive, T-cell depleted allo-BMT. We found that the percentage of CD26- and CD3-positive cells, as well as the levels of expression of both antigens, was lower than in normal controls during the first 4 months after CD6-depleted allo-BMT. Subsequently, the number of lymphocytes expressing CD3 and CD26 and the quantitative surface expression of CD3 and CD26 were not significantly different in patients and normal controls. Functional studies showed that CD26-mediated T-cell proliferation via the CD3 pathway was considerably improved and almost reached normal levels by 1 year, whereas recovery of CD26-mediated T-cell proliferation via the CD2 pathway was delayed for at least 2 years after CD6-depleted allo-BMT. As CD26 involvement in the regulation of human thymocyte activation is restricted preferentially to the CD3 pathway—unlike its involvement with both CD3 and CD2 pathways of peripheral T cells—our results suggest that the different effects of CD26-mediated costimulation via the CD3 and CD2 pathways after CD6-depleted allo-BMT may be a reflection of peripheral T-cell immaturity in those individuals, similar to that seen in mature medullary thymocytes or cord T lymphocytes.

A LLOGENEIC bone marrow transplantation (allo-BMT) has been shown to provide potentially curative therapy for patients with a wide variety of diseases, including many hematologic malignancies. Patients who have undergone allo-BMT are subjected to a variety of infectious complications in the months to years after engraftment. The basis for this increased susceptibility to infections is multifactorial, with immunosuppressive medications, graft-versus-host disease (GVHD), and intrinsic T-cell dysfunction all making significant contributions. Previous studies have documented a variety of in vitro T-cell dysfunctions after allo-BMT, including impaired ability to provide help to B-cell immunoglobulin synthesis, poor proliferative response to mitogenic stimuli, and abnormal function of cytotoxic effector cells. Moreover, these impaired in vitro T-cell functions have been shown to continue for several years, suggesting that these T-cell dysfunction(s) may contribute to increased susceptibility to opportunistic infections for prolonged periods.

The CD3/T-cell receptor (TcR) complex plays a central role in T-cell activation and function. In general, specific peptide antigen or antibodies against the CD3/TcR complex alone cannot induce T-cell proliferation and lymphokine secretion. Rather, T cells require a second costimulatory signal that can be provided by a number of accessory molecules such as CD28, CD29/ActL, CD2, and CD26, which are expressed on the surface of the T cell.

CD26 is a 110-kD glycoprotein present on T cells and was first described as a T-cell activation antigen defined by the monoclonal antibodies (MoAbs) TAL and 1F7. The expression of CD26 is increased after activation of T cells, although it is expressed on a subset of CD4 memory T cells in a resting state. In addition, the CD4, CD6 T-cell subset responds maximally to recall antigens, such as tetanus toxoid (TT). CD26 has been shown to have dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) enzyme activity in its extracellular domain, which cleaves amino-terminal dipeptides with either L-proline or L-alanine in the penultimate position. Considerable evidence suggests the involvement of CD26 in T-cell activation and function. Crosslinking of CD26 with CD3 or CD2 can induce T-cell costimulation and interleukin-2 (IL-2) production by peripheral human CD4 T cells in the absence of antigen-presenting cells.

Although human thymocytes also express CD26, CD2, and CD3, CD26 involvement in the regulation of human thymocyte activation is restricted primarily to the CD3 pathway. This costimulatory activity of CD26 has also been confirmed using a CD26-transfected Jurkat human T cell line that secretes IL-2 after stimulation. The cDNA sequence of CD26 predicts a type II membrane protein with only six amino acids in the cytoplasmic region, suggesting that other associated molecules are involved in CD26-mediated signal transduction. A partial explanation of the mechanism of CD26-mediated T-cell signaling appears to be its association with CD45 phosphatase, as well as with adenosine deaminase (ADA).

To determine whether CD26-mediated T-cell costimulation is involved in prolonged peripheral T-cell dysfunction after allo-BMT, we examined the expression of CD26 and CD26-mediated T-cell costimulation via CD3 and CD2 pathways at various times after CD6-positive, T-cell-depleted allo-BMT. These studies demonstrated that CD26-mediated T-cell proliferation via the CD3 pathway gradually improved.
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and almost reached normal levels by 1 year post-BMT, whereas recovery of CD26-mediated T-cell proliferation via the CD2 pathway was delayed for at least 2 years after CD6-depleted allo-BMT. The different effects of CD26-mediated costimulation via the CD3 and CD2 pathways after CD6-depleted allo-BMT may be a reflection of T-cell immaturity in these individuals similar to that seen in mature medullary thymocytes or cord lymphocytes.

MATERIALS AND METHODS

Patients and samples. This study is based on the analysis of 26 patients who underwent CD6-depleted allo-BMT at the Dana-Farber Cancer Institute (DFCI; Boston, MA). Eleven patients had acute myelogenous leukemia (AML), three had acute lymphocytic leukemia (ALL), and 12 had chronic myeloid leukemia (CML). Ages ranged from 18 to 58 years (median, 35 years). The preparative regimens of allo-BMT have been described previously.25–27 The donor bone marrow was obtained from HLA-identical siblings that ranged from 18 to 58 years (median, 35 years). The preparative regimens of allo-BMT have been described previously.25–27 The harvest of marrow cells was performed with anti-CD6 MoAb (T12, IgM) and rabbit complement before infusion to deplete mature T cells. as described previously.25–27 None of the patients included in this analysis developed moderate or severe graft-versus-host disease (GVHD; grade II to IV), and none were receiving any immunosuppressive therapy. Informed consent was obtained from all patients and normal controls. Peripheral blood was obtained at various intervals post-BMT. A total of 45 samples from allo-BMT patients were analyzed, and a total of 20 samples from age- and sex-matched healthy volunteers were used as normal controls.

MoAbs. MoAbs reactive with CD2 (T11), IgG1, T11/101d2-4C1, IgG2a, T11/1 mono 2A6, IgG3), anti-CD3 (RW28C8, IgG1:OKT3, IgG2A); CD26 (IF7, IgG1), and L-selectin (LAM-1: TQ, IgG1) were produced in our laboratory, and their characterization has been described elsewhere.28–30 Anti-CD2, CD3, CD26, and L-selectin MoAbs used in proliferation studies were purified from ascites using protein-A sepharose beads (Bio-Rad, Richmond, CA). Phenotypic analysis. Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient sedimentation. PBMC were incubated at 4°C with a panel of MoAbs for 30 minutes. Cells were subsequently washed twice and incubated at 4°C with fluorescein isothiocyanate (FITC)-conjugated MoAbs for 30 minutes. Cells were subsequently washed twice and incubated at 4°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (TAGO, Burlingame, CA). Reactivity of MoAbs was analyzed on the gated lymphocyte populations using an Epics-C flow cytometer (Coulter Electronics, Hialeah, FL), as described previously.25–27

Preparation of coimmobilized anti-CD3 MoAb plus additional antibodies. Purified anti-CD3 MoAb (100 μL) at 0.05 μg/mL in phosphate-buffered saline (PBS) was plated in each well of a 96-well flat-bottomed microtiter plate (Costar, Cambridge, MA), which was then incubated overnight at 4°C as described.20 After washing twice with PBS, 100 μL of PBS containing the indicated amounts of additional MoAbs [anti-CD2 (T11), CD26, and L-selectin (CD62-L)] were then plated in each well and incubated overnight at 4°C. Control studies using direct FITC-conjugated anti-CD3 and direct FITC-conjugated anti-CD3 plus direct phycoerythrin-conjugated antibodies recognizing other accessory molecules (CD2 [T11], CD26, and CD62-L) showed that binding of anti-CD3 was independent of the concentrations of the antibodies recognizing other cell surface molecules, and vice versa (data not shown).

Preparation of immobilized anti-CD26 and CD26-mediated T-cell costimulation via the CD2 pathway. Purified IF7 (CD26; 100 μL) MoAb or purified CD62-L MoAb at indicated concentrations was placed in each well of a 96-well flat-bottom microtiter plate (Costar, Cambridge, MA), which was then incubated overnight at 4°C as described.20 Before use, wells were washed twice with PBS. For experiments determining comitogenic effect of solid-phase immobilized anti-IF7 on the CD2-dependent pathway, cells were incubated in the presence of suboptimal doses of the soluble pair of anti-CD2 antibodies recognizing the T112 and T111 epitope (1:4,000 ascites dilution).

Proliferation assays. Lymphocytes were obtained from PBMC by depletion of contaminating monocytes by adherence to plastic plates. Further removal of monocytes was achieved by incubation with 5 mmol/L L-leucine methyl ester HCl as described.32,33 Fresh normal thymus was obtained from patients less than 3 years old who had a part of their thymuses removed at corrective cardiac surgery. A portion was finely minced and a single-cell suspension was prepared by pressing the fragments through a stainless steel mesh. Red blood cells and debris were then removed by centrifugation over Ficoll-Hypaque, and the thymocytes were washed three times. The lymphocyte populations thus obtained were cultured at 37°C in triplicate at a concentration of 105 cells per well in a serum-free medium consisting of Iscove’s Modified Eagle’s Medium (MEM) supplemented with 0.1% bovine serum albumin (BSA), 30 μg/mL human transferrin, 10 μg/mL soybean lipids, 50 μg/mL cholesterol, 4 mmol/L L-glutamine, 0.5% 25 mmol/L HEPES buffer (Microbiological Associates, Bethesda, MD), sodium bicarbonate, and 50 μg/mL gentamicin-sulfate (Schering, Kenilworth, NJ) as described.32,33 For experiments involving the soluble pair of anti-CD2 MoAbs recognizing the T112 and T111 epitopes, solutions containing these antibodies were added at the beginning of the culture period at the asites of 1:4,000 dilutions. Each well contained 1 × 105 lymphocytes or thymocytes, and incubation was performed at 37°C in a 5% CO2-humidified atmosphere. In case of inducing thymocyte proliferation, exogenous human recombinant IL-2 (IL-2; gift from Immunex Corp, Seattle, WA) was added to wells at a final concentration of 20 U/mL as described previously.31 After 3 days in culture, each well was labeled with 1 μCi of [3H]thymidine (ICN Radiochemicals, Irvine, CA) for 18 hours. Cells were then harvested on a PHD cell harvester (Cambridge Technology, Cambridge, MA), and [3H]-thymidine incorporation was measured with a β scintillation counter.

Statistical analysis. Results are expressed as means ± SEM or means ± SD. Comparisons of groups were analyzed using the Mann-Whitney U test. Values of P < .05 were considered significant.

RESULTS

Expression of CD3 and CD26 on lymphocytes post-allo-BMT. To determine the functional properties of CD26 on lymphocytes post–CD6-positive, T cell-depleted allo-BMT, we first examined the expression of CD26 in comparison with that of CD3. As summarized in Table 1, the percentage of CD26- and CD3-positive cells and the levels of expression of both antigens was lower than in normal controls during the first 4 months after CD6-depleted allo-BMT. However, such expression returned to normal levels by 4 months after CD6-depleted allo-BMT. We previously reported that VLA-β1, as well as VLA-α4, α5, and α6 expression, was lower than in normal controls during the first 4 months after allo-BMT and autologous BMT (auto-BMT), whereas expression of these antigens also returned to normal levels by 4 months after allo-BMT and auto-BMT.25 Thus, recovery of CD26 expression after CD6-depleted allo-BMT was similar to that of VLA molecules and CD3.25–26 The mean fluorescence intensity (MFI) of CD26 antigen, as expressed on T cells observed from CD6-depleted allo-BMT patients after 4 months.
post-BMT, was also identical to that seen with normal T cells. (MFI of normal controls, 55.3 ± 10.4 vs that of allo-
BMT patients, 54.0 ± 6.8). However, during the first 4 months after allo-BMT, MFI of CD26 was always higher in normal controls than in CD6-depleted allo-BMT patients (less than 4 months MFI, 40.2 ± 9.0). This finding is in contrast to MFI of the VLA β1 expression on lymphocytes after CD6-depleted allo-BMT, which was higher than that in normal controls.

**Table 1. CD3 and CD26 Expression on Lymphocytes in Blood in Patients With CD6-Depleted Allo-BMT and Normal Control Subjects**

<table>
<thead>
<tr>
<th>Months Post-BMT</th>
<th>CD3</th>
<th>CD26</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 (n = 9)</td>
<td>17.3 ± 12</td>
<td>9.2 ± 6.5</td>
</tr>
<tr>
<td>1-4 (n = 10)</td>
<td>45.2 ± 15.6</td>
<td>23.9 ± 9.1</td>
</tr>
<tr>
<td>4-12 (n = 15)</td>
<td>68.8 ± 12.1</td>
<td>41.3 ± 10.2</td>
</tr>
<tr>
<td>12-24 (n = 10)</td>
<td>73.4 ± 9.6</td>
<td>45.6 ± 7.2</td>
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<tr>
<td>24 (n = 7)</td>
<td>74.1 ± 4.0</td>
<td>49.5 ± 6.3</td>
</tr>
<tr>
<td>Control (n = 20)</td>
<td>75.0 ± 5</td>
<td>49.8 ± 7</td>
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Results are expressed as mean percentages ± SD.

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**Figure 1. Impaired comitogenic effect of solid-phase immobilized anti-CD26 on CD2-dependent T-cell proliferation post-CD6-depleted allo-BMT.**

A) Purified lymphocytes (1 x 10^5 per well) obtained from a healthy volunteer (A) and a patient 12 months post-CD6-depleted allo-BMT (B) were stimulated in a serum-free medium with immobilized anti-CD26 (0.05 μg/mL) alone or with coimmobilized anti-CD3 MoAb and different MoAb (1 and 5 μg/mL) against CD2 (T112), CD26, or L-selectin (LAM-1, CD62-L) or submitogenic doses of the soluble pairs of anti-CD2 (T112 and T111) MoAbs alone or soluble pairs of anti-CD2 (T112 and T111) MoAbs and different MoAb (1 and 5 μg/mL) against CD26 or L-selectin (CD62-L), as described in Materials and Methods. After 3-day culture, proliferative response was assessed by [3H]thymidine incorporation. Results are expressed as the mean counts per minute (cpm) of triplicate samples ± SEM.

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**Figure 2. Summary of results of T-cell proliferation studies obtained from normal controls and patients at various times after CD6-depleted allo-BMT.** When lymphocytes were examined less than 4 months after CD6-depleted allo-
BMT, any combination of anti-CD3 and other MoAbs or that of soluble pairs of anti-CD2 plus other MoAbs could not induce T-cell proliferation. This result indicates dysfunction of both CD3- and CD2-mediated signal pathways, as well as of CD26-mediated costimulation. However, when lymphocytes were examined in patients after 4 months post-
CD6-depleted allo-BMT, CD3/CD2- or CD3/CD26-mediated T-cell proliferation was considerably improved and almost reached normal levels by 1 year post-BMT. In contrast, delayed recovery of CD2/CD26-mediated T-cell proliferation was observed. As shown Fig 2, this impaired T-cell proliferation lasted for at least 2 years after CD6-depleted allo-BMT.

Results of similar studies examining the comitogenic effect of anti-CD26 on CD3- and CD2-mediated pathways of human thymocyte proliferation are shown in Table 2. Although the addition of immobilized anti-CD26 has a comitogenic effect on anti-CD3-mediated thymocyte proliferation, the addition of solid-phase immobilized anti-CD26 did not result in any significant increase in human thymocyte proliferation in the presence of the soluble pair of anti-CD2 MoAbs recognizing the T112 and T111 epitopes. These studies confirm that solid-phase immobilized anti-CD26 has a comitogenic effect on thymocyte activation induced by anti-
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CD3 but not anti-CD2 and are, therefore, functionally similar to peripheral lymphocytes obtained 4 months to 2 years after allo-BMT.

**DISCUSSION**

Previous studies of immune function after allo-BMT have documented a variety of in vitro T-cell abnormalities after engraftment. In our earlier studies, we evaluated cells from patients without GVHD who have received donor marrow depleted of CD6+ T lymphocytes and showed that stimulation by either nonspecific lectin (phytohemagglutinin; PHA) or antibodies to CD2 (T112 and T111) MoAbs resulted in decreased levels of T-cell proliferation compared with controls for over 12 months post-BMT. Yamagami et al reported that CD26 has a comitogenic effect on thymocyte activation induced by anti-CD3 but not anti-CD2. Gerli et al also reported that CD26 has a regulatory role in the activation of cord blood T cells via the CD3/TCR complex rather than through the CD2 activation pathway. However, it is not known whether the prolonged CD26-mediated signal defect via the CD2 pathway in T cells observed after CD6-depleted allo-BMT may be a reflection of the immaturity of allo-BMT patients’ T-cells, similar to that seen with either cord blood T lymphocytes or mature medullary thymocytes.

It should be noted that we examined CD26-mediated T-cell costimulation after allo-BMT in a series of patients who had received HLA-compatible sibling marrow grafts purged of T cells with anti-CD6 MoAb and complement for prevention of GVHD. As a result, none had severe GVHD (grades II to IV), and none were given immunosuppressive medications pre- or post-BMT. Thus, both of these factors did not affect the results of T-cell costimulation, and defective CD26/CD2 costimulation is more likely to reflect an intrinsic T-cell abnormality rather than exogeneous factors.

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**Table 2. IF7 Is Comitogenic With CD3-Mediated Pathway But Not With CD2-Mediated Pathway of Human Thymocyte Proliferation**

<table>
<thead>
<tr>
<th>IF7 Concentration (µg/mL)</th>
<th>[3H]Thymidine Incorporation (cpm x 10^3)</th>
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<tr>
<td>Media</td>
<td>4.5</td>
</tr>
<tr>
<td>1F7</td>
<td>4.9</td>
</tr>
<tr>
<td>1F7/5 µg/mL</td>
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</table>

Proliferation assays were performed as described in Materials and Methods. Immobilized anti-CD3 were used at 0.05 µg/mL and soluble pairs of anti-CD2 (T112 and T111) MoAbs were used at 1:4,000 dilutions of ascites. Exogenous IL-2 was added to all wells at a final concentration of 20 U/mL. Values reported represent the mean of triplicate wells, with SEM as <15%.
CD6-positive, T cell-depleted allo-BMT can be generalized to all allo-BMT patients, because the possibility remains that the difference in the function of CD26 in T cells from T cell-depleted allo-BMT patients and normal controls may be due to the absence or presence of mature T cells in the marrow grafts.

CD26 is a 110-kd T-cell activation antigen that has been shown to have DPP IV enzyme activity, which cleaves aminopeptidase IV activity with either L-proline or L-alanine at the penultimate position. Recent studies have shown that CD26 plays an integral role in T-cell activation. A partial explanation of the mechanism of CD26-mediated T-cell signaling appears to be its association with CD45 tyrosine phosphatase and with adenosine deaminase (ADA). The precise mechanism of the failure of anti-CD26 to affect the CD2-induced activation pathway of T cells from allo-BMT patients is not clear. In cord blood T cells, anti-CD26 (1F7) treatment induces modulation of CD26 but does not comodulate with CD45, suggesting that CD26 is not physically linked to the signal-inducing molecules that may be involved in the activation process. Thus, it is conceivable that the CD26 molecule on allo-BMT patients' T cells may not be appropriately associated with either CD45 phosphatase or ADA. We are now undertaking further experiments to examine this hypothesis. The abnormal CD26 function could explain, at least in part, the persistent immune incompetence status found in patients after allo-BMT and may be involved in susceptibility to opportunistic infections in such patients. Further studies are required to clarify the precise molecular basis for this defect of CD26-mediated costimulation via the CD2 pathway in peripheral T-cells after allo-BMT.

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