Reconstitution of T-Cell Function After CD6-Depleted Allogeneic Bone Marrow Transplantation

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Patients who undergo allogeneic bone marrow transplantation (BMT) are clinically immunodeficient for a prolonged period after engraftment. In the present study, we examined immune function after BMT in a series of patients who had received HLA compatible sibling marrow grafts purged of T cells with anti-CD6 monoclonal antibody and complement. None of the patients in this analysis received immunomodulating agents and none had developed graft-versus-host disease (GVHD). Initially after BMT, natural killer (NK) cells are the predominant cell type, giving way to CD3+ CD6+ T cells after 4 to 8 weeks. Despite the return of normal numbers of T lymphocytes post-BMT phenotypic analysis reveals several long-term abnormalities, including an inverted T4:T8 ratio and a significant fraction of CD3+ T cells that do not co-express CD6. In mitogenic assays, stimulation by either nonspecific lectin (phytohemagglutinin; PHA) or antibodies to the CD2 surface structure (anti-T11, + anti-T11,) results in decreased levels of T-cell proliferation compared with controls for over 18 months post-BMT. In contrast, the ability of unstimulated peripheral blood mononuclear cells (PBMC) to respond to recombinant interleukin-2 (rIL-2) is relatively intact, most likely reflecting early functional reconstitution of the NK cell population. To further characterize the prolonged abnormalities in T-cell proliferation after PHA or CD2 stimulation, we examined more proximal events in T-cell activation such as induction of IL-2 receptor expression and stimulus-induced intracellular calcium flux. We found that the induction of IL-2 receptor (p55) after in vitro activation, although initially abnormal, recovers completely by 6 months post-BMT. We also found that, after CD2 stimulation, calcium flux in T cells was normal immediately after engraftment. In contrast, after stimulation with anti-CD3 antibodies, a large population of T cells do not develop intracellular calcium flux compared with controls. We conclude that despite the recovery of normal numbers of T lymphocytes early after engraftment of CD6-depleted marrow, these T cells exhibit several physiologic and functional abnormalities that persist for varying intervals post-BMT.

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METHODS

Patient population. This study is based on the analysis of 40 patients who underwent allogeneic BMT over a 4-year period at Dana Farber Cancer Institute (DFCI). The transplant protocol was approved by the Institutional Review Board at DFCI. Informed consent was obtained from all patients. Fifteen patients had acute myelogenous leukemia (AML), eight had acute lymphocytic leukemia (ALL), 13 had CML, and four had other hematologic malignancies. Ages ranged from 18 to 55 years (median, 35). The preparative regimen consisted of cyclophosphamide (60 mg/kg) on 2 successive days followed by total body irradiation (TBI). In most patients, the total TBI dose was 1,400 cGy delivered in seven equal fractions. The preparative regimen consisted of cyclophosphamide (60 mg/kg) on 2 successive days followed by total body irradiation (TBI). In most patients, the total TBI dose was 1,400 cGy delivered in seven equal fractions.

In a typical run, 1 x 10^6 cells/mL were processed at a flow rate of 1,000 cells per second for 10 minutes. Anti-CD3 (T3/2Ad2) or anti-CD2 antibodies were added at 30 seconds into the run. Linear integrated signals were displayed as a ratio of long-shit calcium-bound Indo-1 (calcium free-Indo) wavelength as a function of time. The fraction of total CD5+ cells with a fluorescence ratio value of less than 0.5 (the minimal value of the resting state) was calculated at discrete 30 second intervals after the addition of stimulus. The intervals in which the greatest percentage of CD5+ cells had increments in intracellular calcium concentration after the addition of antibody were compared in patients and normal controls by using the Coulter EPICS Extended Analysis computer system program "Cube."

RESULTS

Phenotypic analysis of PBMC after BMT. Immune reconstitution post-BMT was first evaluated by phenotypic characterization of circulating mononuclear cells at regular intervals over a 4-year period. In the first month after marrow infusion, cells bearing the natural killer (NK)-associated antigen, NKH1 (CD56), comprise the predominant mononuclear cell population in peripheral blood. The number of T lymphocytes steadily increases post-BMT so that these cells predominate after 5 to 6 weeks (Fig 1). By 10 weeks post-BMT, the number of T cells in patients approximates that found in normal controls. Although the relative fraction of NK cells in peripheral blood decreases over time, the absolute number actually increases in the first 6 weeks and remains at or above control levels for several months post-transplant. B cell recovery is slow, with only small numbers of CD20+ lymphocytes detected in peripheral blood for the first 3 to 4 months post-transplant. By 6 months post-BMT, 5% to 10% of PBMC in most patients are CD20+. Monocyte reconstitution appears to be quite rapid, with cells expressing CD14 (Mo2) comprising 30% to 40% of PBMC in the first 2 to 3 months after engraftment (data not shown).

Although the overall T-cell compartment appears to reconstitute fairly early after engraftment, it is clear that its composition remains abnormal for greater than 2 years. The T4+ T8 ratio remains well under 1.0 for the first 24 months and is persistently inverted in 50% of patients after 2 years (data not shown). Also, it appears that after CD6-depleted marrow transplantation, a sizable fraction of CD3+ T cells lack the CD6 surface antigen. The CD3+ CD6- cell...
observed this difference in cells from patients who have undergone autologous or syngeneic transplantation. There is a considerable discrepancy in the frequency of expression of these two surface antigens that remains present as late as 24 months after BMT (Fig 2). We have not observed this difference in cells from patients who have undergone autologous or syngeneic transplantation.

**T-cell proliferation after BMT.** Immune reconstitution following BMT was also evaluated by periodic assessment of T-cell function in vitro using a variety of stimuli. Proliferative capacity after exposure as a standard mitogen (PHA) was tested in cells from patients at frequent intervals. As shown in Fig 3A, there was a profound decrease in proliferative potential initially post-BMT, which did not normalize after 18 months. Figure 3B demonstrates that when stimulation of an alternate pathway of T-cell activation was tested with antibodies to the CD2 structure (anti-T11, + anti-T11), a similar phenomenon was observed. In order to determine whether this defect might have been related to decreased availability of IL-2, these experiments were also carried out with the addition of rIL-2 (100 units/mL). Augmentation of both PHA- and CD2-induced proliferation was consistently seen with exogenous rIL-2. However, rIL-2 could only restore thymidine incorporation to normal levels in cells from patients at least 18 months post-BMT.

**Induction of IL-2 receptor expression.** The IL-2 receptor itself can exist in several forms that can mediate ligand binding under low, intermediate, or high affinity conditions. The high affinity IL-2 receptor is a heterodimer composed of a 55 Kd chain (p55) and a 75 Kd chain (p75). It is generally not detected on normal resting T cells in peripheral blood, but its expression is induced after T-cell activation. The 55 Kd and the 75 Kd chains may exist in isolated form on the cell surface. When not associated with the 75 Kd chain, p55 binds IL-2 with low affinity and appears to be nonfunctional.

Using an antibody that recognizes p55 (IL-2R FITC), we observed that after 72 hours of incubation with either PHA or antibodies to CD2, the majority of normal T cells will express this receptor. However, as shown in Fig 4, a significantly smaller fraction of T cells early post-BMT (less than 3 months) will express p55 when similarly stimulated. In contrast to either PHA- or CD2-induced proliferation, full recovery of stimulus-induced p55 expression occurs by 6 months post-BMT.

**Response of resting PBMC to IL-2.** IL-2 itself in the absence of other stimuli can have direct effects on the proliferation of PBMC. When we compared levels of DNA synthesis induced by rIL-2 alone (100 U/mL) in cells from patients and controls, we found no significant differences except in the first 3 months post-BMT. PBMC collected from patients during this period did not respond as well to rIL-2 stimulation as did controls (Fig 5). In order to determine if this difference could be accounted for by a difference in the expression of IL-2R on fresh unstimulated PBMC, we used flow cytometry to assay for the expression of the p55 chain that forms part of the high affinity IL-2 receptor (CD25).

We found no significant differences in the expression of IL-2R by this method (data not shown). However, it is possible that differences in low level expression of p55 actually exist, but that detection of this expression was below the level of sensitivity of flow cytometry. Alternatively, there may be differences in the constitutive expression of the intermediate affinity p75 IL-2 receptor between these populations, thereby explaining the discrepancy in the level of proliferation in response to rIL-2. In order to characterize better the cells responding to rIL-2 alone without other activation signals, we examined the phenotype of proliferating cells after 10 to 14 days of in vitro incubation at 37°C with rIL-2 (100 U/mL). This analysis revealed that greater than 50% of proliferating cells were CD56+, and thus had an NK cell phenotype.

**Intracellular calcium flux after in vitro activation.** The addition of monoclonal antibodies to either the T cell receptor/CD3 complex or the CD2 structure leads to the
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movement of calcium into the cytoplasm from both the extracellular environment and mitochondria. This phenomenon has most often been demonstrated with homogeneous populations of T cells, usually clonal in origin. Recently, the use of photosensitive dyes and flow cytometric techniques has allowed examination of specific subpopulations within larger heterogeneous populations of cells without extensive prior purification of these cells. Using these methods, we have compared the capacity of T cells from both normal individuals and BMT patients to increase intracellular calcium concentrations in response to triggering with anti-CD2 or anti-CD3 antibodies. We accomplished this by gating on PBMC that had been prelabeled with a PE-conjugated CD5 antibody (T1-RDI), which selectively labels virtually all T cells in peripheral blood and was found not to influence calcium mobilization.

As shown in Fig 6A, anti-CD2 antibodies elicited a characteristic response pattern in normal CD5+ cells, which is seen as a 3-minute lag with 70% to 75% of T cells demonstrating appreciable change in intracellular calcium by 10 minutes. Also shown in Fig 6A (bottom panel), the majority of CD5+ cells from patients post-BMT behaved similarly. The interval after BMT did not seem to influence the extent of calcium flux, and CD2 stimulation resulted in normal calcium flux in 4 of 5 patients examined less than 3 months post-BMT. The preservation of calcium mobilization, one of the earliest steps in T-cell activation, contrasts sharply with the delayed recovery observed in BMT patients with respect to both stimulus-induced IL-2 receptor expression and cellular proliferation. Figure 6A also shows that a small proportion of CD5− cells appear to flux calcium upon stimulation with anti-T111+ anti-T111−, most notably in cells from patients less than 3 months post-BMT. By repeating these experiments with cells prelabeled with the NK cell surface marker, NKH1-RDI, we demonstrated that this activity occurs in the NK cell fraction (data not shown).

When we examined calcium flux after stimulation of the CD3 structure, we found that approximately 67% of CD5+
Fig 6. Assessment of calcium flux after stimulation with either CD2 or CD3 antibodies post-BMT. PBMC were loaded with Indo-1 and subsequently surface labeled with CD5-PE antibodies as described in Methods. After 30 seconds of equilibration, antibodies to CD2 (A) or CD3 (B) were added to prepared cells from controls (top panels) or patients (bottom panels). An increase in intracellular calcium concentration in individual cells is represented by a decrease in the long:short wavelength fluorescence ratio. Calcium flux was assessed simultaneously in both T-cell (CD5+) and non-T-cell (CD5-) fractions.

Fig 7. Comparison of intracellular calcium flux after stimulation through CD2 and CD3 pathways. The fraction of CD5+ cells demonstrating a significant increase in intracellular calcium (defined by a drop in fluorescence ratio below 0.5) is displayed in patients and controls after stimulation with antibodies to CD2 (A) and CD3 (B). Fifteen patients and controls were examined. The median fraction of CD5+ cells responding is represented by the horizontal bar.

**DISCUSSION**

It is generally accepted that patients who have undergone allogeneic bone marrow transplantation are immune deficient for months to years after engraftment despite the early recovery of granulocytes and normal numbers of circulating lymphocytes. Numerous abnormalities of T-cell function post-BMT have previously been described, but studies characterizing these defects have frequently been done using cells from patients who either have GVHD or are taking immunosuppressive medication, making accurate assessment of intrinsic T-cell function more difficult. Allowances for these confounding variables can be largely eliminated by examining recipients of T-depleted allogeneic marrow grafts. These patients seldom develop GVHD or need to take additional immunosuppressive agents. Consequently, any defects of T-cell function uncovered in this population of patients are more likely to be intrinsic to the reconstitution process and less likely to be influenced by extrinsic factors.

In the present study, we have evaluated cells from patients without GVHD who have received donor marrow depleted of CD6+ T lymphocytes. We specifically chose to target this surface antigen because it is expressed on mature T cells but not on natural killer cells or other lymphoid populations.

We examined recovering PBMC to determine if manipulation with anti-CD6 antibody significantly alters the phenotypic composition of these cells. We found that the predominant circulating lymphoid population detected in the first 4 to 6 weeks after infusion of CD6-depleted marrow is comprised of NKH1 (CD56) positive cells. The majority of these cells appear morphologically as large granular lymphocytes and do not co-express the CD3/T cell receptor complex. The early recovery of NK cells, both phenotypically and functionally, has been noted in several other studies with both modified and unmodified allogeneic marrow. In fact, the rapid reconstitution of NK cells soon after BMT has even been observed despite transplantation of marrow that has been purged by methods (eg, Campath-1 and soybean lectin agglutination plus E rosette depletion) that eliminate NK...
cells as well as T lymphocytes from the donor marrow inoculum.\textsuperscript{22,23}

Compared with the findings of other studies characterizing immune reconstitution after allogeneic transplantation, we observed no significant delay in the recovery of normal numbers of T lymphocytes.\textsuperscript{22-25} The number of CD3 + T cells increases gradually post-BMT, becoming the predominant lymphoid population by 6 weeks and reaching control levels after 10 to 12 weeks. Phenotypic analysis of T cell subsets reveals a few noteworthy findings. There exists an inversion of the CD4:CD8 ratio that persists in the majority of our patients for more than 2 years, somewhat longer than what has been observed in other reported series.\textsuperscript{22-23} In addition, a significant fraction of CD3+, CD8 + T cells do not express the CD6 surface antigen for a considerable interval post-BMT. Thus, although there appears to be an early return of normal numbers of T lymphocytes after engraftment of CD6 depleted bone marrow, the composition of the T-cell compartment is abnormal and may in part reflect the specific depletion of CD6 + T cells.

Despite normalization of the number of circulating T cells 3 months after engraftment, we found markedly depressed proliferative responses to stimulation with either PHA or anti-CD2 that had not recovered by 18 months post-BMT. Abnormal responses to PHA lasting 4 to 18 months have been documented in other series of purged and unmanipulated transplants.\textsuperscript{9,10,15} However, only scant data have been accumulated on the integrity of the alternate pathway of T-cell activation that involves the CD2 surface structure. T cells can be activated through stimulation of CD2 in the absence of specific antigen, and it has been suggested that the CD2 molecule may play an important role in cell-cell interactions, as well as in cytotoxicity.\textsuperscript{16,28,29} CD2 may also be intricately involved in the regulation of CD3/T cell receptor triggering by antigen.\textsuperscript{30} In conjunction with a previous report that also demonstrated proliferative abnormalities in CD2 activation after conventional non–T-depleted transplantation,\textsuperscript{31} our findings suggest that abnormal function of this pathway is an intrinsic defect inherent to the process of immune reconstitution after BMT and may contribute significantly to immune deficiency post-transplant.

The ability to interact with IL-2 may be an important functional parameter of T-cell recovery post-BMT. In our studies, addition of rIL-2 was able to augment the proliferative response to both PHA and anti-CD2, but not fully correct them, indicating that the proliferative defects observed with these stimuli alone cannot be attributed entirely to inadequate IL-2 production. In order to better understand the interaction between IL-2 and PBMC post-BMT, we examined the expression of IL-2 receptor in these cells after in vitro activation, using an antibody to the 55 Kd chain of the receptor. This chain may be expressed individually in a nonfunctional form on the cell surface or may be associated with a 75 Kd chain to form a high affinity receptor. After in vitro stimulation with PHA or anti-CD2, we found diminished expression of p55 on T cells from patients less than 6 months post-BMT. Although the antibody we used cannot distinguish between the nonfunctional single chain receptor and the high affinity heterodimer, our results suggest that, within the first 6 months post-BMT, the inability to express IL-2R after activation may contribute significantly to the markedly abnormal proliferative responses to PHA and anti-CD2. Beyond 6 months post-BMT, defects in proliferation still occur despite a degree of surface p55 induction equivalent to that seen in controls. In these patients, a greater fraction of the detected p55 may exist as the nonfunctional solitary chain and not as the high affinity functional receptor.

The role of IL-2 in immune reconstitution post-BMT was also evaluated by the analysis of the proliferative response to this cytokine alone in the absence of additional stimulation. Since very few resting T cells in peripheral blood express either the high or intermediate affinity IL-2R, only a very small number are capable of responding to rIL-2 alone. These T cells are presumably ones that have undergone activation in vivo. In contrast, it has recently been shown that the majority of resting NK cells constitutively express isolated intermediate affinity IL-2R (p75). Thus, NK cells are capable of responding to rIL-2 without prior stimulation.\textsuperscript{32,33} After CD6-depleted allogeneic transplantation, the ability of PBMC to proliferate in response to rIL-2 was essentially preserved with only a modest reduction noted in the first 3 months after engraftment. This response is likely to reflect functional NK cell reconstitution, since phenotypic analysis of PBMC from patients after 10 to 14 days of culture in rIL-2 (100 U/mL) reveals that NK cells undergo significantly greater expansion than do T cells. It is of note that at the concentration of rIL-2 that we used (100 U/mL or 435 pmol/L), a significant proportion (~40%) of the p75 intermediate affinity binding sites are occupied on normal NK cells.\textsuperscript{34} The diminished response we observed to rIL-2 alone in the first 3 months post-BMT could conceivably reflect differences in the expression of the p75 receptor and is currently under investigation in our laboratory.

Evaluation of T cell function after bone marrow transplantation has traditionally focused on phenotypic, proliferative, and functional parameters. Examination of physiologic processes crucial to cellular activation has not been extensively undertaken. In our CD6-depleted marrow recipients, we explored one of the initial events in the activation of T cells, generation of a transmembrane calcium flux. When T cells are incubated with either anti-CD2 or anti-CD3 antibodies, there is a relatively rapid influx of extracellular calcium along with the hydrolysis of phosphatidylinositol 4.5 biphosphate (PIP2) by phospholipase C, therupon releasing inositol triphosphate (IP3) and diacylglycerol (DG).\textsuperscript{35} IP3 acts as a second messenger mediating further intracellular calcium release.\textsuperscript{36} DG serves to activate protein kinase C (PKC) and stimulate its translocation from the cytosol to the cell membrane.\textsuperscript{37} Although CD2-induced proliferation is abnormal post-BMT, we found no differences in calcium flux between controls and patients, including those less than 3 months after engraftment. This result suggests that the effect in CD2-associated functional response is located distal to the initiation of calcium flux and may be found later in signal transduction, perhaps related to the activity of PKC. Indeed, Izquierdo et al recently reported a defect in the Na+/H+ antiporter-mediated alkalinization of intracellular pH, an
early cellular metabolic event associated with PKC activation.  
We repeated our calcium studies with an antibody to the CD3 surface molecule and found, in contrast to CD2 stimulation, considerable differences between patients and controls in the fraction of CD5+ cells that could respond with a change in intracellular calcium. This discrepancy did not significantly change with time post-BMT and could not be overcome by the addition of more antibody. It is unclear why a significant proportion of post-BMT T cells do not appear to respond to anti-CD3 antibodies with a flux of calcium. It is possible that the CD5+ cells on which we gated were not all T cells and, therefore, did not co-express CD3.

There have been reports of CD5 expression on circulating B lymphocytes after BMT. However, in several patients tested, greater than 90% of CD5+ cells are CD3+ T cells. More importantly, the CD5+ cells of our patients flux calcium normally in response to anti-CD2 antibodies, a stimulus to which B cells would not respond. Calcium flux after anti-CD3 stimulation has been evaluated in recipients of conventional marrow grafts by Lopez-Botet et al. They found no abnormalities in calcium mobilization in several patients tested. Although it is possible that their anti-CD3 antibody recognized different antigenic determinants or had different functional properties than did ours, it is intriguing to speculate on whether the contrast in the two results could be related to phenotypic differences in the recovering T cells. After CD6-depleted BMT, we observe a significant fraction of CD3+ cells that do not co-express CD6. Recent studies have suggested that the CD6 molecule may play a role in T-cell activation, and thus, CD3+, CD5+, CD6− T cells may represent a functionally distinct subset of T cells that are unresponsive to CD3 stimulation.

Although our results must be interpreted in the context of the specific T-cell depletion process we used, it is clear that distinct functional and physiologic parameters recover at different intervals post-BMT in patients who do not have GVHD and are not receiving immunosuppressive medica-

tions. For example, proliferation in response to PHA or anti-CD2 is abnormal for extended periods despite early phenotypic T-cell reconstitution. In contrast, the ability to induce IL-2 receptor (p55) expression by these same stimuli recovers far sooner (by about 6 months), while calcium flux after triggering with anti-CD2 antibodies is normal by 4 to 6 weeks after engraftment. Interestingly, a subpopulation of T cells that can flux calcium in response to anti-CD2 cannot do so after anti-CD3 stimulation. It remains uncertain which of these tests of T-cell function have important clinical implications. Despite a similar reversal of the T4:T8 ratio, as well as an abnormal proliferative response to mitogenic stimuli seen in our recipients of purged autologous transplants, differences are apparent between these two groups in their susceptibility to certain infectious agents (e.g., cytomegalovirus) early post-BMT. Other in vitro tests of T cell function, such as induction of IL-2R by different stimuli, could conceivably be a more important yardstick with which to measure immune recovery than are proliferation assays or measurements of T4:T8 ratios.

The importance of expanded investigation on immune reconstitution after T-depleted transplants is underscored by the controversy that exists over the potential adverse consequences of purging marrow to eliminate GVHD. Several groups have reported increases in graft failure, relapse rates, and opportunistic infections in patients who received T-depleted marrow compared with those given unmanipulated grafts. Understanding the recovery of T-cell function post-BMT could help identify precisely what specific cellular defects are associated with these clinical sequelae. Additionally, since not all groups have experienced these adverse phenomena and since methods of purging vary between institutions, immune reconstitution studies may help to explain the differences in the reported results. By further studies of the reconstitution of the immune system after BMT, we may ultimately be able to design protocols that can simultaneously minimize the risks of GVHD without promoting tumor recurrence or infectious complications.

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