Proliferation and Cytolytic Function of Anti-CD3 + Interleukin-2 Stimulated Peripheral Blood Mononuclear Cells Following Bone Marrow Transplantation

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We evaluated the proliferation, cytolytic function, and phenotypic characteristics of anti-CD3 plus interleukin-2 (IL-2) stimulated peripheral blood mononuclear cells (PBMCs) from 44 patients with leukemia or non-Hodgkin’s lymphoma (NHL) treated with multagent chemotherapy or following bone marrow transplantation (BMT). BMT patients had decreased cell growth with only a 1.35 ± 0.25 (autologous BMT for acute lymphoblastic leukemia [ALL]), 1.24 ± 0.25 (autologous BMT for NHL), and 0.8 ± 0.1 (allogeneic BMT for leukemia) mean fold increase by day 5 of culture compared with controls (4.0 ± 0.4), P < .001. Anti-CD3 + IL-2 activated cells from patients with ALL and NHL who had received autologous BMT and cells from patients with leukemia who underwent allogeneic BMT were more effective in lysing the natural killer (NK) sensitive target, K562, and the NK-resistant target, Daudi, compared with controls. In contrast, cytolyis of K562 and Daudi by cultured PBMCs from patients with ALL and NHL receiving multi-agent chemotherapy was similar to that of controls. Cultures from BMT recipients had a significant increase in CD16 (autologous ALL 5.7 ± 1.5%, P < .01; autologous NHL 12.4 ± 3.5%, P < .001; allogeneic 14.3 ± 2.9%, P < .001) and CD56 cells (autologous ALL 27.8 ± 12.0%, P < .01; autologous NHL 39.3 ± 9.5%, P < .001; allogeneic 42.7 ± 7.4%, P < .001) compared with controls (CD16 2.5 ± 0.4%; CD56 6.9 ± 0.9%). Stimulation of PBMCs with anti-CD3 + IL-2 is effective in generating cells with high cytolytic function post-BMT.

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Adoptive transfer of IL-2 stimulated cells in combination with systemically administered IL-2 has been associated with the regression of melanoma and renal cell carcinoma in humans and in therapeutic responses of a large variety of murine tumors. Recent trials have shown complete or partial responses in patients with advanced lymphoma after systemic administration of IL-2 with or without adoptively transferred cells.

We and others have shown that the anti-CD3 (OKT3) monoclonal antibody (MoAb) can be used in conjunction with IL-2 in vitro to stimulate peripheral blood mononuclear cells (PBMCs) to acquire LAK activity. This method also results in large increases in cell numbers in both human and murine systems compared with conventional IL-2-stimulated cultures. Furthermore, on a per-cell basis, cytolytic activity against various targets is comparable to that of IL-2-stimulated cells. In the current study we have shown that anti-CD3 + IL-2 activated cells from autologous or allogeneic BMT recipients have enhanced cytolytic function compared with those from healthy controls and from patients receiving multiagent chemotherapy for leukemia and lymphoma. These results indicate that LAK function can be increased in the early post-BMT period and that anti-CD3 + IL-2 stimulated cells may be useful for adoptive transfer in patients with lymphoid malignancies undergoing BMT.

MATERIALS AND METHODS

Patients. Forty-four patients (22 male, 22 female) with ages ranging from 5 to 68 years (median 37 years) were studied. Seventeen healthy volunteers were used as controls. Six patients with acute lymphoblastic leukemia (ALL) and 10 with non-Hodgkin’s lymphoma (NHL) had received multiagent chemotherapy within the year before study, while seven ALL and nine NHL patients underwent autologous BMT. An additional 12 patients with various hematologic malignancies (six acute nonlymphoblastic leukemia [ANLL], three chronic myelogenous leukemia, two ALL, one chronic lymphocytic leukemia) received allogeneic BMT. Pretransplant conditioning included total body irradiation and high-dose cyclophosphamide plus additional etoposide (VP-16) for five patients. Five of the allogeneic BMT patients received T-depleted marrow with T101 MoAb (anti-CD5) linked to ricin immu-
were drawn at 28 to 159 days (95 ± 20) after BMT in the autologous group and 22 to 201 days (92 ± 18) in the allogeneic group (P = not significant).

**Cell preparation and culture.** Heparinized blood was diluted 1:1 with Hank's Balanced Salt Solution 1X (HBSS) (GIBCO, Grand Island, NY) and layered over PicoHypaque (PharmaCia, Piscataway, NJ). Interface PBMCs were washed three times in tissue culture media (TCM), consisting of RPMI 1640 (GIBCO) supplemented with 25 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin (Sigma, St Louis, MO), and 5% pooled heat-inactivated human serum. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in 75-cm² flasks (Corning, Corning, NY) at a concentration of 0.5 x 10⁶ cells/mL in TCM with 10 ng/mL anti-CD3 MoAb (OKT3; Ortho, Raritan, NJ) and recombinant IL-2 100 U/mL (Hoffmann La Roche, Nutley NJ; specific activity 1.5 x 10⁶ U/mg) or IL-2 alone. Anti-CD3 MoAb was added on the first day of culture only. Cells were subcultured to 0.2 to 0.4 x 10⁶ cells/mL in TCM containing IL-2 when they exceeded 1 x 10⁶ cells/mL.

**Estimation of cell number increase and viability.** Cultures were sampled on day 5 and 7 of culture; 10 μL of culture aliquots were mixed with 10 μL of trypan blue (GIBCO) and counted using a standard hemocytometer. Viability of cells was consistently greater than 90%.

**Tumor lines.** Human tumor cell lines, K562 (NK-sensitive; erythroleukemia cell line) and Daudi (NK-resistant; Burkitt lymphoma cell line) were maintained in culture with TCM and subcultured at 0.5 to 1 x 10⁶/mL in fresh media two to three times per week.

**Cytotoxicity assay.** Tumor cell line targets (K562, Daudi) (1 to 2 x 10⁶ in 0.5 mL TCM) were incubated with 500 μCi Na¹⁵CrO₄ (5,000 μCi/mL) (New England Nuclear Research Products, Boston, MA) for 1 to 1½ hours at 37°C. Targets were washed three times in cold TCM and resuspended at a concentration of 1 x 10⁶ cells/mL. Fifty microliters (500 target cells) were then added to 96-well V-bottomed microtiter plates (Costar, Cambridge, MA) into which effectors (day 5 anti-CD3 + IL-2 or IL-2 stimulated PBMCs) had been previously added in triplicate and serially diluted in TCM to yield effector to target ratios (E:T ratio) of 100:1, 33:1, 11:1, and 3:1. Spontaneous release wells contained only TCM and ¹⁵Cr-labeled targets while maximal release wells contained detergent and targets. The microtiter plates were gently centrifuged at 50g for 5 minutes and incubated at 37°C and 5% CO₂ for 4 hours. The plates were then centrifuged at 150g for 10 minutes after which 100-μL aliquots of supernatant were harvested into glass scintillation vials (Dyanalon, Rochester, NY) and 2.5 mL of scintillation fluid added (Cytoscreen; ICN Biomedicals, Irvine, CA). Radioactivity was counted using an LKB 1216 liquid scintillation counter. Cytotoxicity was determined by the formula:

\[
\% \text{ Cytotoxicity} = 100 \times \frac{\text{Experimental Mean cpm} - \text{Spontaneous Release Mean cpm}}{\text{Maximal Release Mean cpm} - \text{Spontaneous Release Mean cpm}}
\]

One lytic unit (LU) was defined as the number of effectors required to lyse 30% of targets; cytotoxicity is presented as LU per 10⁶ effector cells.

**Phenotypic analysis by immunofluorescence.** Fresh PBMCs or cells in culture for 5 days with anti-CD3 + IL-2 were washed in phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal calf serum and 0.1% sodium azide (Sigma) and 1 x 10⁶ cells were placed in 96-well V-bottomed microtiter plates. MoAbs used for double-color analysis included FITC-conjugated or PE-conjugated MoAbs anti-Leu-4 (CD3), anti-Leu-3 (CD4), anti-Leu-2 (CD8), anti-Leu-11 (CD16), anti-Leu-19 (CD56), and anti-IL-2 R (CD25) (Becton Dickinson, Mountain View, CA). Double-color analysis was performed by incubating 10 μL of FITC-conjugated and PE-conjugated MoAbs with the cells for 30 minutes at 4°C. The cells were then washed three times with PBS containing 2% fetal calf serum and 0.1% sodium azide and fixed with PBS containing 2% paraformaldehyde. Analyses was performed using a Becton Dickinson FACScan.

**Statistical analysis.** The Student's t-test was used to compare growth, percent cytolysis, and immunophenotype of cells. Statistical comparisons of the distribution of lytic units between groups were made using the nonparametric Mann-Whitney U test.

**RESULTS**

**Comparative proliferation and cytolytic function of IL-2 and anti-CD3 + IL-2 stimulated cells after BMT.** PBMCs from 11 patients who underwent BMT (seven autologous BMT for NHL, two autologous BMT for ALL, and two allogeneic BMT for ANLL) were obtained to evaluate if the addition of anti-CD3 to IL-2 increases cell proliferation after BMT. Samples were taken 20 to 180 days (93 ± 31) after transplant. The mean fold increase in cell number of anti-CD3 + IL-2 stimulated cultures on day 7 was significantly greater (3.5 ± 1.0) than cultures stimulated with IL-2 alone (0.5 ± 0.1) (P < .01) (Fig 1). Percent cytolysis at 33 to 1 effector to target ratio was comparable with a mean ± S.E.M. of 65.1% ± 3.4% (IL-2 stimulated cultures) versus 63.7% ± 2.1% (anti-CD3 + IL-2 stimulated cultures) against K562 target (P = not significant) and 63.0% ± 9.3% versus 68.2% ± 7.2% against Daudi target (P = not significant). Due to the superior growth of anti-CD3 + IL-2 stimulated cultures, all further experiments in this report were performed using this method of cell activation.

**Proliferation of anti-CD3 + IL-2 stimulated cells.** Anti-CD3 + IL-2 stimulated PBMCs from patients with ALL treated with chemotherapy had significantly less growth after 5 days of culture with a 2.0 ± 0.5 mean fold increase compared with 4.0 ± 0.4 in controls (P < .02) (Fig 2). Proliferation of PBMCs from NHL patients who received multiagent chemotherapy (3.5 ± 0.6) did not differ from that of controls. Bone marrow transplant patients had a strikingly blunted cell growth when compared to controls with only a 1.35 ± 0.25 (autologous BMT for ALL), 1.24 ± 0.25 (autologous BMT for NHL), and 0.8 ± 0.1 (allogeneic BMT for leukemia) mean fold increase by day 5 of culture, P < .001 (Fig 2).

**Generation of cells with cytolytic effector function.** To determine the cytolytic function of day 5 anti-CD3 + IL-2 stimulated cells, cytotoxicity against NK-sensitive K562 and NK-resistant Daudi cell lines was assessed using a 4-hour ⁵¹Cr release assay. The cytolytic function of effectors from BMT recipients was generally higher than controls. In contrast, cytotoxic function of cells derived from patients treated with chemotherapy was similar to controls (Fig 3). Due to the similar cytotoxicity demonstrated by cultures from ALL and NHL patients receiving chemotherapy and the comparable cytotoxicity between cells from ALL and...
Fig 1. Comparative mean fold increase in cell number between IL-2 and anti-CD3 + IL-2 stimulated cultures on day 7 of culture. PBMCs were stimulated with IL-2 100 U/mL or activated with anti-CD3 MoAb (OKT3 10 ng/mL) on day 0 and placed in TCM containing 100 U/mL IL-2. The mean fold increase in cell number of anti-CD3 + IL-2 stimulated cultures on day 7 was significantly greater (3.5 ± 1.0) than cultures stimulated with IL-2 alone (0.5 ± 0.1). (P < .01). Data expressed as mean ± SEM.

Fig 2. Mean fold increase in cell number on day 5 of culture. PBMCs were activated with anti-CD3 MoAb (OKT3 10 ng/mL) on day 0 and cultured in TCM containing 100 U/mL IL-2. PBMCs from patients with ALL treated with chemotherapy had significantly less growth after 5 days of culture compared to controls (P < .02). Proliferation of PBMCs from NHL patients who received multiagent chemotherapy (chemo) did not differ from that of controls. Bone marrow transplant patients had significantly less cell growth than controls (auto BMT for ALL, NHL and allo BMT for leukemia); all P < .001. Data expressed as mean ± SEM.

NHL recipients of autologous BMT, patients were combined into chemotherapy (chemo) and autologous BMT (auto BMT) groups for statistical analysis (Mann-Whitney U test; Fig 3). Cytotoxicity of effectors from patients receiving chemotherapy was not significantly different from that of controls; however, cultured cells from autologous and allogeneic BMT recipients showed a significant increase in cytolytic function (K562; auto BMT P < .001, allo BMT P < .01) (Daudi; auto BMT P = .05, allo BMT P = .06). Similarly, the percent cytotoxicity of effectors from patients receiving chemotherapy was not significantly different from that of controls (t-test); however, cultured cells from autologous and allogeneic BMT recipients showed a significant increase in cytolytic function (Table 1). There was no association between the time interval from chemotherapy or BMT to initiation of anti-CD3 + IL-2 cultures and degree of cytolytic function (data not shown).

Phenotype of fresh PBMC and anti-CD3 + IL-2 stimulated cells. As shown in Table 2, the proportion of CD3+ PBMCs did not differ significantly between controls and patients treated with chemotherapy but BMT patients had a significantly lower percentage of CD3+ cells. This was due to a decreased number of CD4+ cells, resulting in an inverted CD4/CD8 ratio post BMT. After 5 days in culture with anti-CD3 + IL-2, cell cultures from BMT recipients continued to have a lower percentage of CD3+ cells. The proportion of CD16+ and CD56+ in cultured PBMCs was significantly higher in patients receiving BMT compared with controls. Expression of the p55 IL-2 receptor (CD25) was infrequent in all groups' fresh cells. After stimulation with anti-CD3 + IL-2 for 5 days, CD25 expression was similar in controls and chemotherapy-treated patients but significantly lower in patients after BMT (Table 2).

DISCUSSION

There has recently been increased interest in investigation of immunotherapy regimens after BMT in an attempt to decrease disease recurrence. Enhancement of the immune response against cancer at a time of minimal residual disease may be a promising adjuvant to high-dose chemotherapy and total body irradiation as currently used in BMT. Recent studies have demonstrated the presence of circulating cells with LAK activity in the early posttransplant period. IL-2 stimulation of these cells augmented their capacity to lyse tumor targets. Others have shown...
that IL-2 stimulated cells can be effective in purging marrow of neoplastic cells.\textsuperscript{28,29}

We have previously reported on the use of anti-CD3 MoAb (OKT3) in combination with IL-2 to generate rapidly proliferating cultures of cells with LAK activity.\textsuperscript{14,15} These culture conditions resulted in an average 1,000-fold increase in cell number over 21 days compared with less than 100-fold increase when IL-2 was used alone. In this report we first compared proliferation of PBMCs from BMT patients cultured with IL-2 to those activated with anti-CD3 + IL-2 (Fig 1). Cultures containing IL-2 alone showed very poor growth; therefore, we decided to assess the effectiveness of anti-CD3 + IL-2 stimulation in generating cells with LAK activity post BMT. Using this approach we have evaluated the proliferation, cytolytic function, and phenotype of PBMCs from BMT recipients and compared these findings with those of healthy controls and with patients receiving multi-agent chemotherapy but not BMT. Proliferation of anti-CD3 + IL-2 stimulated PBMCs from patients receiving autologous or allogeneic BMT was reliable but lower than that of controls (Fig 2). However, the cytolytic function on a per-cell basis of day 5 cultured cells

<table>
<thead>
<tr>
<th></th>
<th>K562</th>
<th>Daudi</th>
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<tr>
<td>Controls</td>
<td>44.9 ± 3.1</td>
<td>58.6 ± 2.7</td>
</tr>
<tr>
<td>Chemotherapy ALL</td>
<td>50.5 ± 9.9</td>
<td>55.8 ± 11.0</td>
</tr>
<tr>
<td>Chemotherapy NHL</td>
<td>57.0 ± 6.8</td>
<td>55.5 ± 9.5</td>
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<tr>
<td>Autologous BMT ALL</td>
<td>64.7 ± 6.0</td>
<td>71.1 ± 5.1</td>
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<tr>
<td>Autologous BMT NHL</td>
<td>63.6 ± 1.7</td>
<td>76.3 ± 5.9</td>
</tr>
<tr>
<td>Allogeneic BMT</td>
<td>60.3 ± 5.6</td>
<td>73.8 ± 7.1</td>
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Shown are the mean ± SEM cytolysis of the respective target at 33:1 effector to target ratio.

Abbreviation: NS, not significant.
from transplant patients was enhanced compared with controls (Table 1). This is in contrast to the report by Higuchi et al.8 in which LAK cells generated postautologous BMT did not have higher cytolytic function when compared with those from healthy controls. This data may suggest that anti-CD3 + IL-2 activation may be a promising technique to generate larger numbers of potent cytolytic effectors for adoptive immunotherapy post BMT.

Fresh PBMCs from healthy volunteers and patient groups studied had a similar number of CD16+ and CD56+ cells. However, after 5 days in culture BMT patients had a significantly higher number of CD16+ and CD56+ cells (Table 2). It has been reported that CD16+ and CD56+ cells mediate most of the LAK activity in IL-2 stimulated cultures.13,15 We have previously reported that at least three different cell subpopulations develop LAK activity in long-term cultures with anti-CD3 + IL-2: (1) CD3+, CD16+; (2) CD3+, CD16+, CD56+; (3) CD3+, CD4+, CD8+.15 In this study we did not evaluate the contribution of each cell subpopulation to total LAK activity generated. However, given the previous data it appears that the higher cytolytic function of cultured PBMCs from BMT patients was related to their higher percentage of CD16+ and CD56+ cells.

Thus, anti-CD3 + IL-2 activation appears to be a promising technique to generate cells that may be used in an adoptive immunotherapy program as adjuvant therapy in post BMT patients. Further studies are needed to define signals beyond anti-CD3 and IL-2 that may improve proliferation of these cells posttransplant while retaining their enhanced lytic function.

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**REFERENCES**


6. Weisdorf DJ, Nesbit ME, Ramsay NKC, Woods WG, Gold-


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E Katsanis, PM Anderson, AH Filipovich, DE Hasz, ML Rich, CM Loeffler, AC Ochoa and DJ Weisdorf