Autologous Peripheral Blood Stem Cell Transplantation and Adoptive Immunotherapy with Activated Natural Killer Cells in the Immediate Posttransplant Period

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ABSTRACT

Relapse after high-dose chemotherapy supported by peripheral blood stem cell transplantation (HDC-PBSCT) is the main cause of therapeutic failure in patients with lymphoma and breast cancer. Adoptive immunotherapy with activated natural killer (A-NK) cells and interleukin 2 might eliminate surviving residual tumor without adding to toxicity.

Eleven patients with relapsed lymphoma and one with metastatic breast cancer were entered on a pilot clinical trial of HDC-PBSCT followed on day 2 after transplant by infusion of cultured autologous A-NK cells. Simultaneously, recombinant human interleukin 2 (rhIL-2) was initiated as a 4-day continuous i.v. infusion at 2 \times 10^5 IU/m^2/day, referred to as high-dose rhIL-2. Therapy with high-dose rhIL-2 was followed by a 90-day continuous i.v. infusion at 3 \times 10^5 IU/m^2/day, referred to as low-dose rhIL-2. All patients engrafted and nine completed treatment. Posttransplant days to a neutrophil count of 500/\mu l and to a platelet count of 50,000/\mu l were similar to comparable patients treated with HDC-PBSCT alone. Generation of A-NK cells for therapy was feasible in all patients except the three patients with Hodgkin’s disease, whose cells did not proliferate in culture. Overall toxicity associated with early posttransplant transfer of A-NK cells and interleukin 2 did not differ from that observed with peripheral blood stem cell transplantation alone in comparable patients. There was early amplification of natural killer cell activity in the peripheral blood of four patients that appeared to result from the transfused A-NK cells. Adoptive transfer of A-NK cells and rhIL-2 during the pancytopenic phase after HDC-PBSCT was feasible and well tolerated, did not adversely affect engraftment, and resulted in amplified natural killer activity in the peripheral blood during the immediate posttransplantation period.

INTRODUCTION

The use of HDC3 and hemopoietic stem cell support has become widely employed in the treatment of relapsed lymphoma and metastatic breast cancer (1–7). In chemotherapy-sensitive lymphoma and metastatic breast cancer, HDC can provide disease-free survival at 5 years in 40% and 15% of remitting patients, respectively. Nevertheless, the majority of completely remitting patients will relapse and die of their disease. Dose escalation of chemotherapy and radiation therapy above conventional transplant doses has not improved disease-free survival.

AIT with in vitro-activated autologous effector cells, either LAK cells or tumor-infiltrating lymphocytes, in combination with IL-2 has been shown to induce long-term responses in a small proportion of patients with metastatic melanoma or renal cell carcinoma (8, 9). These two types of cancer are generally chemoresistant but seem to be among the most responsive to biological therapy. Experience with the therapeutic use of cellular effectors and IL-2 in lymphoma or breast cancer has been limited, and only patients with advanced disease have been treated (10). Nevertheless, a few responses have been seen (11), indicating that AIT might be effective in these diseases. The treatment protocol described here is an attempt to evaluate the feasibility of AIT with A-NK cells and IL-2 in the setting of HDC-PBSCT in patients with lymphoma or breast cancer at the time of maximum chemotherapy effect.

A-NK cells are a subset of CD3-CD56dim peripheral blood NK cells selected by adherence to plastic in the presence of 22 nm of IL-2 (12). A-NK cells can be expanded in culture with IL-2 for 2 to 3 weeks (12). This subset of NK cells has been shown recently to preferentially enter human tumor spheroids or established human tumor xenografts in nude mice and to kill NK-resistant tumor cells (13). In murine models of tumor metastasis, systemically delivered A-NK cells have been shown to preferentially localize to metastases (14). Our hypothesis has

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been that A-NK cells mediate significant antitumor effects when administered systemically and home to tumor metastases growing as solid tissue. Thus, systemic infusion of A-NK cells supported with IL-2 might eliminate residual tumor surviving HDC in patients with advanced cancer and decrease the relapse rate following HDC-PBSCT. As a first step in testing this hypothesis, this study demonstrates the feasibility of generating A-NK cells in patients with advanced lymphoma and of combining HDC-PBSCT with AIT using A-NK cells and IL-2 during the cytopenic phase immediately after the transplant.

### MATERIALS AND METHODS

#### Patients
All patients gave informed consent for participation in the AIT protocol, which was approved by the Pittsburgh Cancer Institute and the Institutional Review Board of the University of Pittsburgh. The patient characteristics are listed in Table 1. Six women and six men were entered on the study. The mean age of the patients was 44 (range, 26-61) years. Eight patients had lymphoma, three had Hodgkin’s disease, and one had metastatic breast cancer. Compassionate approval was obtained for treatment of the breast cancer patient. These patients received transplants between November 1992 and February 1994. All patients had failed at least one chemotherapy regimen. Five of the 12 patients entered on this protocol were judged to have chemotherapy-responsive disease, demonstrated as a partial or complete response to ifosfamide chemotherapy on evaluation 4–8 weeks after its administration.

The patients participating in this study were compared to patients on the AIT protocol, which was approved by the Pittsburgh Cancer Institute and was under review by the Food and Drug Administration. The following Monday, daily 4-h leukapheresis began and continued for 4 days (second leukapheresis series). The time from the first day of leukapheresis for PBSCs (first leukapheresis) varied between 2 and 4 weeks. The WBC was normal at the time of the second leukapheresis procedure. The comparison targeted toxicity and engraftment, looking for clinically relevant differences.

#### Treatment Protocol
The treatment schema for the lymphoma patients is shown in Fig. 1. Patients were admitted to hospital and had a Hickman pheresis catheter placed in the subclavian position under local anesthesia. Chemotherapy consisting of ifosfamide and Mesna at 3 g/m²/day by continuous i.v. infusion for 4 and 5 days, respectively, for the lymphoma patients and cyclophosphamide and mesna at 5 g/m² by continuous i.v. infusion for 1 and 2 days, respectively, for the patient with breast cancer was given. Following chemotherapy, G-CSF (Neupogen; Amgen, Thousand Oaks, CA) at 5 µg/kg/day s.c. was administered. Daily blood counts were performed. On the first week day, after ifosfamide-induced leukocyte nadir, with WBC >1000/µl, leukapheresis for collection of PBSC was started. A total of 7–9 × 10⁸ MNC/kg were collected.

On the first Monday after PBSCT collection, rhIL-2 (Chiron Corp., Emeryville, CA) at 3 × 10⁶ IU/m²/day by continuous i.v. infusion was started, using a Pharmacia-Deltic CADD-1 pump (Pharmacia Deltic, St. Paul, MN) and was continued for 6 days. The following Monday, daily 4-h leukapheresis began and continued for 4 days (second leukapheresis series). The time from the first day of leukapheresis for PBSCs (first leukapheresis) and the beginning of leukapheresis for A-NK cells (second leukapheresis) varied between 2 and 4 weeks. The WBC was normal at the time of the second leukapheresis procedure. The collections were processed by the Cellular Adoptive Immunotherapy Laboratory of the Pittsburgh Cancer Institute and were cultured for the generation of A-NK cells as described below.

The patients were then admitted to the Bone Marrow Transplant Unit to begin HDC. Supportive care included single rooms with positive pressure high efficiency particular air filtered air, low microbial content diets, prophylactic antibiotics and acyclovir, blood product support, and i.v. antibiotics and amphotericin as clinically indicated. The patients with lymphoma received transplants between November 1992 and February 1993, with autologous PBSCT for recurrent lymphoma or Hodgkin’s disease on another protocol open at our institution. This protocol targeted the same patient population, and all patients would have been eligible for transplant on the AIT protocol. Reasons for not participating in the AIT protocol were patient refusal, or a period of time, January 1993 to April 1993, when the AIT protocol was not open for accrual, because it was under review by the Food and Drug Administration. All of the 42 patients who received PBSCT alone received G-CSF after transplant to accelerate leukocyte recovery and to potentially reduce the length of hospital stay. Patients on the AIT protocol did not receive G-CSF posttransplant, so as not to mask any adverse effect of AIT on engraftment or to add a confounding variable to the attribution of toxicity posttransplant. The comparison targeted toxicity and engraftment, looking for clinically relevant differences.

#### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age at Transplant (yr)</th>
<th>Diagnosis</th>
<th>Time between diagnosis and transplant (months)</th>
<th>Prior therapy</th>
</tr>
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<tr>
<td>380</td>
<td>46</td>
<td>BRC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56</td>
<td>CMF (6), ROTE, ADRIA (3)</td>
</tr>
<tr>
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<td>60</td>
<td>FML</td>
<td>32</td>
<td>CHOP (10)</td>
</tr>
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<td>385</td>
<td>49</td>
<td>DSCCL</td>
<td>12</td>
<td>ProMACE-CYTABOM (6), CNO (2)</td>
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<tr>
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<td>49</td>
<td>FML</td>
<td>50</td>
<td>ProMACE-CYTABOM (2), DICE (3)</td>
</tr>
<tr>
<td>448</td>
<td>42</td>
<td>DLCL</td>
<td>12</td>
<td>CHOP (6), ProMACE (1)</td>
</tr>
<tr>
<td>459</td>
<td>26</td>
<td>HD-NS</td>
<td>43</td>
<td>MitoxBVD (8), ROTE, MOPP/ABV</td>
</tr>
<tr>
<td>463</td>
<td>29</td>
<td>HD-NS</td>
<td>46</td>
<td>MOPP-ABVD (12)</td>
</tr>
<tr>
<td>465</td>
<td>27</td>
<td>HD-NS</td>
<td>32</td>
<td>MOPP (6)</td>
</tr>
<tr>
<td>469</td>
<td>56</td>
<td>FSCCL-DML(T)</td>
<td>61</td>
<td>CHOP (6)</td>
</tr>
<tr>
<td>475</td>
<td>40</td>
<td>DML</td>
<td>17</td>
<td>ProMACE-CYTABOM (4), ROTE</td>
</tr>
<tr>
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<td>61</td>
<td>FSCCL-DLCL(T)</td>
<td>131</td>
<td>COAMP (6), ROTT</td>
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<tr>
<td>501</td>
<td>61</td>
<td>DLCL</td>
<td>51</td>
<td>COP-BLAM (6), CVP (5)</td>
</tr>
</tbody>
</table>

<sup>a</sup> BRC, breast cancer; FML, follicular mixed lymphoma; DLCL, diffuse large cell lymphoma; HD-NS, nodular sclerosing Hodgkin’s disease; FSCCL-DML(T), transformation (FSCCL to DML); ROTE, limited field radiotherapy; ROTE, extensive field radiotherapy; DML, diffuse mixed lymphoma; DSCCL, diffuse small cleaved cell lymphoma; FSCCL-DLCL(T), transformation (FSCCL to DLCL).

<sup>b</sup> Numbers in parentheses, cycles of chemotherapy given.
Patients received the PBSC product on day 0 and the A-NK cells on day +2, both as a rapid i.v. infusion. On day +2, coincident with the A-NK cell infusion, a continuous i.v. infusion of rhIL-2 at 2 $\times$ 10^6 IU/m^2/day (HD-rhIL-2) was started and continued for 4 days. Thereafter, the dose of rhIL-2 was reduced to 3 $\times$ 10^5 IU/m^2/day (LD-rhIL-2), and IL-2 was continued as outpatient therapy for 90 days.

Clinical evaluations of response were performed at 1 and 3 months posttransplantation and then every 3 months for 2 years. Toxicity grading was performed daily during hospitalization and then weekly during outpatient treatment.

**PBSC Product.** PBSCs were obtained daily by 4-h leukapheresis (Cobe Spectra; Cobe BCT, Inc., Lakewood, CO) until the total collected MNC count was 7-9 $\times$ 10^8/kg. All patients received 7 $\times$ 10^8 MNC/kg, with the excess portion reserved for treatment of graft failure. A median number of seven collections (range, 5-11) was performed per patient. Total cell counts were determined on an automated cell counter (Coulter ZM; Coulter, Hialeah, FL). The pheresis product (50% v/v) was mixed with 10% DMSO (v/v, Cryoserv; Research Industries Corporation, Salt Lake City, UT), and 20% autologous plasma (v/v) in 20% Medium 199 (Life Technologies, Inc., Grand Island, NY). The cell concentration was adjusted to not exceed 2 $\times$ 10^6 cells/ml, and 60-ml aliquots were transferred to bags (Cel Freeze Cryogenic Storage Containers; Chartered, Lakewood, NJ) for controlled rate freezing. The bags were stored in liquid nitrogen and thawed in a 37°C water bath at the bedside immediately prior to infusion.

**A-NK Cell Product.** Following low-dose IL-2 priming (see Fig. 1), leukapheresis was performed daily for 4 consecutive days to collect cells for the generation of A-NK cells. MNC were separated by Ficoll-Hypaque density gradient centrifugation and depleted of monocytes with treatment with 5 mm phenylalanine methyl ester (Terumo Medical Corp., Elkton, MD) for 40 min at room temperature (17). Monocyte depletion was previously found to be necessary for optimal culture of A-NK cells (12). The monocyte-depleted MNC were washed and resuspended at a concentration of 5 $\times$ 10^6 cells/ml in the complete culture medium (RPMI 1640 from GIBCO, Grand Island, NY) containing 6000 IU/ml rhIL-2 (Chiron Corp.), 10% pooled, heated-inactivated human AB serum (v/v; Nabi, Miami, FL), and 50 $\mu$g/ml gentamicin (GIBCO). A-NK cells were captured on the surface of plastic culture flasks during 4-5-h incubation in the presence of 6000 IU/ml of IL-2 in an atmosphere of 5% CO_2 in air at 37°C, as previously described (12). Nonadherent MNC were removed by vigorous washing of the flasks with warm culture medium containing 2% human AB serum (v/v). Fresh complete culture medium containing 6000 IU/ml rhIL-2 was added back to the plastic culture flasks, and the number of cells adherent to plastic determined using an inverted microscope. A-NK cell cultures were supplemented with irradiated allogeneic concanavalin A-pretreated MNC on day 1, as described earlier (12), and cultured for 14-18 days. A-NK cell cultures were monitored three times a week for growth and viability of cells by hemacytometer counts performed with trypan blue dye on aliquots of the culture. Cultures were maintained at a cell density of 1-2 $\times$ 10^6 cells/ml by supplementation with complete culture medium containing rhIL-2 every 4 days. On the day of patient infusion (day +2), aliquots of the A-NK cell product were sampled for flow cytometry, and assessments of cytotoxic activity against K562 and Daudi cell lines as well as bacterial and fungal culture were made. The A-NK cell product was characterized by purity of the cells (% CD3+ CD56+), fold expansion in culture based on the initial and final cell counts (12), and cytotoxic activity against the selected tumor cell targets (Table 2).

**Flow Cytometry.** MNC were isolated from the patients' peripheral blood by centrifugation over Ficoll, washed, and incubated with FITC- and PE-conjugated mAbs in preparation for two-color flow cytometry. In some cases, peridinin chlorophyll protein-conjugated mAbs were also used for three-color flow. Isotype controls used were IgG1-FITC, IgG2A-PE, and IgG1-peridinin chlorophyll protein. Gates were set to include

![Fig. 1 Flow diagram of the treatment plan for patients with lymphoma undergoing HDC-PBSCT followed by AIT with A-NK cells and IL-2. CII, continuous i.v. infusion; LD-CII-IL-2, low-dose continuous i.v. infusion of IL-2.](cancercellares.aacrjournals.org)
CD45<sup>−</sup> cells and to exclude debris. The mAb-labeled cells were analyzed with a FACSscan flow cytometer using Consort-32 software (Becton Dickinson, San Jose, CA). The mAbs used for staining were specific for CD3, CD45, CD56 (Becton Dickinson, and CD16 (Medarex, West Lebanon, NH). When cell numbers permitted, 10<sup>4</sup> gated events were analyzed (approximately 90% of samples).

Total numbers of CD34<sup>+</sup> and lineage negative (CD34<sup>−</sup>) cells were determined in the daily PBSC product by flow cytometry. An aliquot of the PBSC daily product was stained with two anti-CD34, FITC-conjugated mAbs (8G12; Becton Dickinson and O bend-10; GenTrak, Plymouth Meeting, PA) and four lineage specific PE-conjugated mAbs with specificity for CD3, CD11b, CD14, and CD19 (Becton Dickinson). U937 cells were used to gate out autofluorescence, and debris was excluded by reference to 6-μm beads. The percentage of each CD34<sup>+</sup> population in 20,000 events was used to quantify the total count of each population in the PBSC product.

**Cytotoxicity Assay.** The 51Cr release assay used in our laboratories has been described in detail previously (18). Briefly, resting NK or A-NK cells were incubated with 51Cr-labeled K562 or Daudi target cells for 4 h at E:T ratios ranging from 6 to 50:1 for resting NK cells and from 1 to 6:1 for A-NK cells. Cytotoxicity was expressed as LU<sub>24/10<sup>7</sup> cells determined in 4-h 51Cr release assays.

**RESULTS**

**Generation of A-NK Cells.** A-NK cells obtained from the peripheral blood of normal volunteers were previously found to proliferate extensively (up to 5000-fold expansion during 14 days of culture) in our hands (12). However, it was uncertain whether it would be feasible to generate these effector cells in patients with lymphoma or metastatic breast cancer. We found that in all patients, it was possible to generate highly purified A-NK cells from leukapheresis products collected after 6-day priming with LD-rhIL-2 (Table 2). In three patients with Hodgkin’s disease, A-NK cells failed to expand in culture under conditions used routinely in our laboratory (Table 2). The levels of proliferation in 14-day cultures of A-NK cells obtained from patients with NHL varied from 1- to 148-fold. Neither the levels of proliferation in 14-day cultures of A-NK cells obtained from patients with HD, as compared to the patients with lymphoma (Table 2). All three patients with Hodgkin’s disease had a nodular sclerosing histology. Extent of prior treatment, time interval between diagnosis and transplant, and disease extent did not predict for the ability to generate A-NK cells in this group of patients. Overall, in 6 of 12 patients, it was possible to obtain >1 × 10<sup>10</sup> A-NK cells forAIT. In 8 of 12 patients, cultured A-NK cells were >85% CD3<sup>−</sup>CD56<sup>+</sup> cells, and the A-NK cell culture with the lowest purity contained 64% CD3<sup>−</sup>CD56<sup>−</sup> cells.

**Engraftment.** All patients engrafted and became transfusion independent following HDC-PBSCT and AIT. The median day to reach an ANC of 500/μl was 14 (range, 11–19) days. The median day to reach a platelet count of 50,000/μl was 13.5 (range, 9–200) days. The median time for granulocyte recovery was 13.5 (range, 9–200) days. The median time for granulocyte recovery was significantly (P < 0.001) shortened in 42 patients treated with G-CSF after HDC-PBSCT relative to 11 patients who received AIT with A-NK cells posttransplant. This difference is consistent with G-CSF administration, suggesting no adverse

**Table 2** Proportions of NK cells and NK activity in the pheresis products used to start the A-NK cell cultures and characteristics of A-NK cells generated for therapy.

<table>
<thead>
<tr>
<th>Pheresis product</th>
<th>A-NK cells</th>
<th>Cytotoxicity vs. K562&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Fold proliferation&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Total no. of cells infused&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CD3&lt;sup&gt;−&lt;/sup&gt;CD56&lt;sup&gt;+&lt;/sup&gt; (%)&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Cytotoxicity vs. Daudi&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>UPN</td>
<td>CD3&lt;sup&gt;−&lt;/sup&gt;CD56&lt;sup&gt;+&lt;/sup&gt; (%)&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
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<td>95</td>
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* Determined by two-color flow cytometry.
* Cytotoxicity expressed as LU<sub>24/10<sup>7</sup></sub> cells determined in 4-h 51Cr release assays.
* Total number of cells on day 14 of culture divided by number of initial plastic-adherent cells.
* Number of therapeutic culture cells infused.
* 1.1 × 10<sup>7</sup> cells were generated from this culture; however, these were not infused because the patient was taken off protocol on day −5 due to the appearance of symptomatic CNS lymphoma.
cells/kg, and in comparison to control patients, whose grafts effect on neutrophil engraftment from AlT. Seven patients had cells/kg. 

\[\text{CD34}^+ \text{cells/kg} \]

Fig. 2 Scatterplot of day posttransplant to an ANC > 500/µl and a platelet count > 50,000/µl in patients undergoing PBSCT with AlT or G-CSF posttransplant. All patients received <6 \times 10^5 \text{CD34}^{+}\text{cells/kg}.

An effect on neutrophil engraftment from AlT. Seven patients had rapid, sustained platelet engraftment and achieved a platelet count of 50,000/µl 1–3 days before their ANC reached 500/µl. Five of these patients received less than 6 \times 10^6 of CD34^{+}\text{cells/kg}, and in comparison to control patients, whose grafts contained similar numbers of stem cells, their platelet engraftment was significantly more rapid (Fig. 2; \( P < 0.001 \)). Our experience has been that regardless of the type of transplant or the source of stem cells, any patient receiving >1 \times 10^7 \text{CD34}^{+}\text{cells/kg} rapidly engrafted, achieving an ANC > 500/µl and a platelet count > 50,000/µl on or before day +13. The same was true for PBSCT, when >6 \times 10^6 \text{CD34}^{+}\text{cells/kg} were transplanted. Therefore, we compared patients who received <6 \times 10^6 \text{CD34}^{+}\text{cells/kg} and reached a platelet count of 50,000/µl before 40 days in both the AlT and G-CSF transplant groups. Those patients who received AlT had significantly accelerated platelet production after transplantation (Fig. 2).

In aggregate, these data indicate that AlT with A-NK cells plus IL-2 did not adversely affect hematopoietic recovery, in a clinically relevant fashion, when administered immediately after PBSCT and may have accelerated platelet engraftment in a subset of patients.

Toxicity. Therapy was discontinued in three patients (UPN 485, 465, and 448 on days +1, +5, and +25, respectively). Reasons for discontinuation were: CNS lymphoma (UPN 448 and 485) and severe anxiety (UPN 465). Both patients with CNS lymphoma were free of symptoms on entry into the study, but developed signs of CNS involvement before A-NK cells and HD-rhIL-2 were administered. One patient (UPN 448) received intrathecal chemotherapy and radiation to the pons for CNS lymphoma before HDC began. She continued on treatment until day +25, when she requested to be withdrawn from the protocol, because of persistent quadraparesis. Four patients have died; UPN 385 on day +109 from interstitial pneumonitis, UPN 448 on day +72 and UPN 485 on day +162 from CNS lymphoma, and UPN 469 on day +171 from recurrent lymphoma.

The choice of A-NK cells for AlT was based on our preliminary data indicating that this subset of IL-2-activated cells is more active in this group of patients. Toxicity from the rhIL-2 and A-NK cells was limited to mild hypotension and edema occurring from days +3 to +7. Two patients received low-dose dopamine for hypotension and had reduced urine output during this time. No patient experienced renal failure, veno-occlusive disease, hemorrhagic cystitis, or diffuse alveolar hemorrhage.

Immunological Modulation. The percentage of CD3^{+}CD56^{+} or CD3^{+}CD56^{+}CD16^{+} (NK) cells and NK activity against K562 targets in the patients’ peripheral blood were measured at various times during the treatment. Table 3 shows the results of NK phenotyping and NK activity in four patients for whom serial data were available before and in the early posttransplant period. Of the four patients, three (UPN 380, 386, and 475) had normal NK activity before therapy. During the period between day +3 and +8, when the absolute leukocyte count was <200/µl, variable but detectable cytotoxicity was observed in the blood, which might be attributable to A-NK cell infusion. Fig. 3 shows serial results in UPN 386, demonstrating the initial peak of NK activity followed by the gradual rise attributed to LD-rhIL-2 over 90 days posttransplant. In general, the number of circulating NK cells after transplant did not correlate with NK activity. As illustrated in Fig. 3, despite a rapid fall in cytotoxicity from the peak on day +3, the number of NK cells continued to increase in the peripheral blood. Similar increases in the absolute number of circulating NK cells were seen in other patients. Spontaneous cytolytic activity of fresh peripheral blood mononuclear cells against Daudi targets was detected in the peripheral blood of two patients (UPN 385 and 386) in the immediate posttransplant period, and this could be interpreted as additional evidence in support of the presence of circulating transferred A-NK cells.

Ifosfamide. Ifosfamide chemotherapy was used to test for chemo sensitivity of each lymphoma and for mobilization of PBSCs. Five of 10 patients responded to ifosfamide (2 complete responses, 3 PR, overall response rate of 50%). No patient exhibited progressive disease on ifosfamide. One patient (UPN 485) developed a Fanconi-like renal wasting syndrome that recovered spontaneously. Outside of predictable pancytopenia, there were no other side effects directly attributable to ifosfamide.

Busulfan/Cyclophosphamide. There was no mortality attributable to the conditioning regimen, and toxicity was transient. The 3-month response evaluations showed that response had improved in five patients, was unchanged in three, and had worsened in two. Three of the five patients experienced improvement from a PR to complete response and two from stable disease to PR. This conditioning regimen was well tolerated and active in this group of patients.

DISCUSSION

This clinical trial demonstrates the feasibility of adding AlT with autologous A-NK cells and IL-2 in the pancytopenic period immediately after PBSCT. This is a rational combination of therapies, because they differ in their mechanism of action, mechanism of disease resistance, and toxicity. In our hands, AlT was used in an effort to exert a further antitumor effect at the time of minimal disease after HDC-PBSCT.

The choice of A-NK cells for AlT was based on the preliminary data indicating that this subset of IL-2-activated

\[\text{ANC} \]

\[\text{ANC} > 500/\mu l\]
Table 3  NK activity and percentage of MNC with NK cell phenotype in the blood of patients treated with HDC-PBSCT and AIT

<table>
<thead>
<tr>
<th>UPN (diagnosis)</th>
<th>Before therapy</th>
<th>Days +3 to +8</th>
<th>Day +90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LU^a</td>
<td>LU^b</td>
<td>% NK cells</td>
</tr>
<tr>
<td>380 (BRC)</td>
<td>319</td>
<td>772</td>
<td>32</td>
</tr>
<tr>
<td>385 (DSCCL)</td>
<td>33</td>
<td>264</td>
<td>2</td>
</tr>
<tr>
<td>386 (FML)</td>
<td>325</td>
<td>1673</td>
<td>41</td>
</tr>
<tr>
<td>475 (DML)</td>
<td>415</td>
<td>287</td>
<td>n/a</td>
</tr>
</tbody>
</table>

^a Only the patients cited exhibited increased NK activity in the blood on days +3 to +8, the other patients had values between 0 and 33 LU (see Table 1 for diagnosis codes).

^b This measurement (LU) was obtained before any protocol therapy had started and at least 4 weeks past any prior therapy.

^K activity was measured in the PBMC of patients using 4-h ^51Cr release assays with K562 as targets and is expressed as LU/10^7 effector cells (LU). The normal range in our laboratory is 50–300 LU, with a median of 132 LU.

^d The absolute leukocyte count was <200/l at the time of this measurement. No lytic activity is detectable in the absence of A-NK cell infusion.

^e % NK cells was determined by flow cytometry in two (CD356) or three-color (CD356 16k) analysis. The normal range for our laboratory is 6–21%, with a median of 12%.

^f The absolute leukocyte count was normal at the time of this measurement.

CD3^-CD56dimCD16^- or dim NK cells have potent antitumor activity in vitro and in vivo (12, 19) and is particularly effective in targeting metastases (14, 20). A-NK cells have been shown to be able to produce a variety of cytokines (20, 21), localize to metastases (20), and rapidly eliminate established metastases in animal tumor models (13, 14, 19). Activities of A-NK cells are strictly dependent on the presence of IL-2 (10, 22). At 2 × 10^6 IU/m^2/day (HD-rhIL-2), the serum concentration of IL-2 was estimated to be sufficient to bind high affinity IL-2 receptor (22). Thus, we reasoned that the infused A-NK cells, a proportion of which expresses the high affinity IL-2 receptor (12), were likely to be stimulated in vivo, at least during the initial 4 days after adoptive transfer.

We hypothesized that the timing of A-NK cell transfusion might be critical for cure. Animal models of combined chemotherapy and AIT have shown that the smaller the time interval between the two therapies, the greater the cure rate (23, 24). On the basis of earlier observations, we planned to give the A-NK cells and IL-2 as close to the chemotherapy as possible.

Our main concerns were the possible adverse effect of AIT on engraftment and the added toxicity of AIT in the pancyclopenic phase after HDC-PBSCT. Our preclinical studies showed no suppression of colony formation in vitro by human A-NK cells and that at the higher A-NK to progenitor ratios, there may actually be enhancement of growth (25). Murphy et al. (26, 27) and Siefer et al. (28) have shown accelerated platelet production after marrow transplantation, in a murine model, that is dependent upon infusion of murine NK cells. We believe that we may have observed an analogous phenomenon in humans.

The administration of IL-2 at doses sufficiently high to support the antitumor activity of A-NK cells was an essential component of the therapy. To reduce IL-2-related toxicity, two design principles were used in this trial. First, the doses of rhIL-2 used were one to two orders of magnitude less than those...
used previously for therapy. Second, during the pancytopenic phase, induction of cytokine secretion from monocytes and thromboxane A2 from neutrophils would be absent. These secondarily generated molecules are thought to mediate the toxic effects of IL-2 (29), and their absence would be expected to reduce IL-2-induced toxicity. Indeed, toxicity attributable to IL-2 was tolerable in this trial. The subsequent 90-day infusion with LD-rhIL-2 was used in the hope of sustaining or expanding in vivo antitumor effector cells. It has been shown previously to expand the number of circulating CD56+ NK cells without affecting the number of T lymphocytes in patients with cancer and prime for LAK activity generated in vitro with higher concentrations of IL-2 (30–32).

The NK and LAK activity observed in the blood of these patients can be explained by the persistence in the circulation of functional transfused A-NK cells. NK activity in the blood is normally absent after transplant and returns no earlier than the third week. In Fig. 3, the initial peak of activity observed at day +3 coincides with the transfusion of the A-NK cells and their support with HD-rhIL-2. The subsequent fall in activity can be ascribed to the death of the A-NK cells, which may at least partially be due to the withdrawal of HD-rhIL-2. The presence of cells with NK phenotype but lack of activity suggests the presence of a nonfunctional precursor that acquires NK activity with maturation. The cells responsible for this activity are probably not of transfused A-NK cell origin.

The ability to consistently generate A-NK cells from the peripheral blood of patients with advanced lymphoma or solid tumors has also been of concern (33). To facilitate A-NK cell generation, a 6-day-long infusion of LD-rhIL-2 before the series of phereses for A-NK cell generation was included in the protocol. The intent was to increase the yield of A-NK cells in culture by priming their progenitors in vivo. Using this approach and the improved culture conditions, i.e., short adherence time and irradiated feeder cells, as described earlier (12), we have been able to generate cell cultures which were highly enriched in CD3–CD56+ cells in all patients with lymphoma. However, A-NK cells obtained from the peripheral blood of the 3 patients with Hodgkin’s disease showed poor in vitro growth. No specific cause for this poor A-NK cell generation in culture could be discerned, and it did not appear to be related to the extent of prior treatment these patients received or to the extent of their disease. Both the number of NK cells in the peripheral circulation and NK activity were normal at baseline in these three patients. Hodgkin’s disease is known to be associated with a profound cellular immune deficit (34), and it is intriguing to speculate that the observed inability to generate A-NK cells might be related to a low number or poor function of A-NK cell precursors in the peripheral blood of these patients.

Using a combination of HDC-PBSCT and AIT with A-NK cells and IL-2 had no clinically relevant adverse effects on engraftment and toxicity, which were both comparable to those seen with HDC-PBSCT alone. In a subset of patients treated with AIT, platelet engraftment may have been accelerated. In addition, early amplification of NK cell function in the cytopenic phase after transplantation, that could have its origin in the transfused A-NK cells, was observed in some patients. Future studies will attempt to optimize AIT with A-NK cells and to dissect the contribution of each transfused cell population to the immunological changes observed.

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