Allogeneic Peripheral Blood Progenitor Cell Transplantation in a Murine Model: Evidence for an Improved Graft-Versus-Leukemia Effect

By Bertram Glass, Lutz Uharek, Matthias Zeis, Peter Dreger, Helmut Löffler, Jörg Steinmann, and Norbert Schmitz

Peripheral blood progenitor cells (PBPCs) are increasingly being used to replace bone marrow cells (BMCs) as a source of hematopoietic stem cells also in the field of allogeneic transplantation. Whereas it is well known that PBPC grafts and BM differ significantly in progenitor cell content and lymphocyte dose, the clinical consequences of these differences with respect to engraftment, graft-versus-host disease (GVHD), and the graft-versus-leukemia (GVL) effect are more difficult to assess. We present a murine model that allows us to evaluate engraftment, GVHD, and GVL effect of allogeneic PBPC transplantation (PBPCT). Balb/c mice (H-2d) served as recipients. Donors were major histocompatibility complex-matched DBA/2 mice or syngeneic Balb/c mice, respectively. Experiments with increasing numbers of BMCs or Filgastrin-mobilized PBPCs showed that the number of progenitor cells in the graft was correlated with the probability to engraft, irrespective of the graft type. With identically high cell numbers transferred (1 x 10^6 nucleated cells/kg body weight [BW]), the mortality rates due to GVHD (25%) were about the same after allogeneic BM transplantation (BMT) and allogeneic PBPC, although PBPC grafts contained four times more CD3+ T cells as compared with BM grafts (6.2 x 10^5 v 1.4 x 10^5/kg BW). For investigation of GVL activity, Balb/c recipients were injected with syngeneic cells of the B-lymphocytic leukemia cell line A20 2 days before transplantation. After total body irradiation to a dose of 7.5 Gy, 10^7/kg BW Balb/c PBPCs, DBA BMCs, or DBA PBPCs were infused. The relapse rates observed were 80% after syngeneic PBPC (n = 22), 60% after allogeneic BMT (n = 23), and 34% after allogeneic PBPC (n = 26) (allogeneic BMT v PBPC, P = .032). We conclude that transplantation of allogeneic PBPCs instead of BM may enhance the GVL effect without an increase of GVHD.

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Allogeneic bone marrow transplantation (BMT) is the treatment of choice for a variety of hematologic malignancies. Animal experiments performed as early as 1957 suggested that the strong antileukemic effect of BMT resulted not only from the high-dose chemoradiotherapy preceding the transplantation but also was a consequence of adoptive immunotherapy exerted by the graft itself. Numerous experimental and clinical reports have confirmed these findings and underscored our view that the superior antitumor effect of BMT is primarily due to the destruction of leukemic target cells by an allogeneic immune response mediated by T lymphocytes and natural killer (NK) cells contained in the transplant.

Recently, granulocyte colony-stimulating factor (G-CSF)—mobilized peripheral blood progenitor cells (PBPCs) have been introduced as an alternative source of hematopoietic stem cells for allogeneic transplantation. Harvesting PBPCs instead of BM avoids the discomfort of marrow harvesting and the risks of general anesthesia in the donor while the high numbers of progenitor cells contained in such a graft lead to rapid and reliable engraftment in the recipient. The large numbers of progenitor cells that can be harvested from the PB 12,13 also allow for extensive ex vivo manipulations and make PBPC attractive as target cells for gene therapeutic purposes and immunotherapy.

Important questions related to PBPCT remain unanswered.

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For smaller grafts, a cell concentration of $1 \times 10^7$ NC/mL was used. BM or PBPC grafts were injected intravenously 2 hours after conditioning of the recipients by TBI.

**T-cell depletion of PBPC grafts.** Immunomagnetic depletion of T cells from the PBPC grafts was used in some experiments. Immunomagnetic beads (Dynal, Oslo, Norway), coated with goat-anti-mouse Ig, were coupled to an antihuman CD3 monoclonal antibody (MoAb; KT-3; Serotec, Wiesbaden, Germany). The CD3 MoAb was used at a concentration of 11 $\mu$g per $4 \times 10^6$ beads. Coupling of MoAb and magnetic beads was performed at 4°C for 30 minutes. The ratio of beads/target cell was 6:1. The magnetic beads were coincubated with the PBPC graft for 1 hour at 21°C, and the cells were then removed with a strong permanent-magnetic device (Dynal). The T-depleted grafts were examined for their content of residual T cells by fluorescence-activated cell sorting (FACS) analysis. The content of T cells after depletion was at the threshold of detection by FACS analysis (1% of nucleated cells; range, 0.2% to 2.3%).

**Progenitor cell colony assays.** For determining the progenitor cell content of the grafts, the Methocult GF M3434 kit was used (Stemcell Technologies, Vancouver, British Columbia, Canada). The assays were performed according to the protocol of the manufacturer. Colony-forming units–granulocyte-macrophage (CFU-GM) were counted 12 days after the plating of $1.5 \times 10^5$ nucleated cells per dish. The cell concentration was $1.5 \times 10^7$ NC/mL. Experiments were performed in parallel to the transplantation experiments with unaltered numbers of PBPCs and BMCs.

**Leukemia cells.** A20 is a B-cell leukemia/lymphoma of Balb/c origin that occurred spontaneously in a 15-month-old mouse. The cells were maintained in culture with RPMI 1640 + 5% fetal calf serum at 37°C and 5% CO2. We performed in vivo passages of these cells by intravenous injection into Balb/c animals. After the mice had developed hepatosplenomegaly, they were killed and their spleens were removed. Spleen cell suspensions containing close to 100% of these in vivo passaged leukemia/lymphoma cells were stored in liquid nitrogen and used for further experiments.

**Experimental design and definitions.** Engraftment and GVHD were investigated in leukemia-free mice of the Balb/c strain. Donors were either animals of Balb/c or of DBA/2 origin. Increasing numbers of BMCs or PBPCs were transferred to the recipients after TBI (7.5 Gy). The animals were examined at least once daily afterwards. Signs of GVHD (weight loss, rough fur, and gibbus) were documented. PB counts were performed every 3 days from day 7 to complete recovery of hematopoiesis or death. In a number of surviving animals, long-term chimerism was investigated by FACS analysis determining the presence/absence of the Lyt 1.1 antigen (CD5, clone H11 86.1; Pharmingen, San Diego, CA) on spleen cells. This antigen exists in two different forms: Balb/c mice express the isoform Lyt 1.2, whereas DBA/2 mice are known to express Lyt 1.1. Animals were killed on day 100 posttransplantation, their spleens were removed, and a single-cell suspension was produced. Untreated animals of Balb/c and DBA/2 origin were used as negative and positive controls, respectively. If the recipient mouse showed the same fraction of splenocytes (≥6%) being positive for Lyt 1.1 as for the DBA control, it was named a long-term chimera. Investigation of the GVL effect was performed in Balb/c recipient animals injected intravenously with $1 \times 10^7$ A20 cells 2 days before transplantation of either $2 \times 10^7$ Balb/c PBPCs, DBA PBPCs, or DBA BMCs, respectively. The animals were examined daily and necropsied after death. The cause of death was determined according to the following definitions. Graft failure or graft rejection was defined as death between day 6 and 30 after transplantation with leukocytes less than 1/mL and granulocytes greater than 0.5/mL. Death due to GVHD was defined as death with recovery of hematopoiesis (leukocytes >3/mL and thrombocytes >50/mL) and macroscopic signs of GVHD such as weight loss, rough fur, hair loss, and gibbus. In some mice with macroscopic GVHD, histologic examination of skin and liver was performed that showed changes compatible with acute GVHD.<ref>Leukemic relapse was defined as death with macroscopic tumor and liver weight more than 1.5 g and spleen weight more than 0.15 g. Histologic examination of liver and spleen was performed for two animals in each group. Animals with hepatosplenomegaly were found to harbor leukemic cells without exception. Healthy mice of the same age have liver weights of 1.3 ± 0.2 g and spleen weights of 0.1 ± 0.02 g, respectively. Statistics. Survival data and freedom from leukemia were calculated according to the method of Kaplan and Meier. The experimental groups were compared using the log-rank test. The calculations were performed on a PC with Statistica statistical software (StatSoft, Tulsa, OK).</ref>

**RESULTS**

**Mobilization of PB progenitor cells and composition of the transplants.** To avoid pooling of PBPCs in the spleen, mice were splenectomized before G-CSF administration. This led to an increase of white blood cells from 6.4/nL to 9.9/nL (range, 9.6 to 10.8 cells/nL) in DBA/2 mice. Differential counts were not altered after splenectomy. G-CSF administration induced a substantial increase of the leukocyte count up to 59.6/nL (range, 42.9 to 73.3/nL) in Balb/c mice or 72.9/nL (range, 51.8 to 85.8/nL) in DBA mice. In steady state, blood of DBA mice contained 0.80 CFU-GM/10^9 NC. After mobilization, the PBPC collection product contained 1.13 CFU-GM/10^9 NC, resulting in a substantially higher concentration of progenitor cells in PB (8.23 CFU-GM/µL after and 0.79 CFU-GM/µL blood before G-CSF administration). BM of DBA mice gave rise to 4.83 CFU-GM/10^9 NC. Balb/c mice were shown to have lower concentrations of progenitor cells in BM or PB, but the relationship between the PBPC graft and BM was identical (Table 1). A total of $2 \times 10^7$ NC/animal was the standard dose transferred to each animal in GVHD and GVL experiments. With the average weight of a Balb/c mouse being 20 g, this would correspond to a dose of $1 \times 10^8$ NC/kg body weight (BW) and $11 \times 10^6$ CFU-GM/kg BW administered with an allogeneic PBPC graft. The progenitor cell dose administered with the standard BMT procedure was fourfold higher. The percentage of lymphocytes was significantly higher in the PB as compared with the BM transplants. Allogeneic PBPC grafts contained 62% (range, 50% to 71%) of CD3+ cells; BM grafts contained 14% (range, 6% to 29.8%; Table 1). This corresponded to $6.2 \times 10^7$ T cells/kg BW for allogeneic PBPC and $1.4 \times 10^8$ T cells/kg BW for allogeneic BMT, respectively, with the standard transplant.

**Engraftment.** In experiments with nonleukemic animals, three parameters possibly determining engraftment were varied: the cell dose of the transplant, the immunogenetic difference between donor and recipient, and the source of hematopoietic stem cells. With PBPCs, the number of nucleated cells transferred had a major impact on engraftment as measured by the rate of graft failure (Fig 1) or the kinetics of hematopoietic recovery in the surviving animals (Fig 2). Transplantation of a higher cell dose was followed by a lower rejection rate and faster hematopoietic recovery after both syngeneic and allogeneic transplantation. Using the same fixed cell dose, the rate of graft failure was higher.
Table 1. Cellular Composition of Stem Cell Transplants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>n</th>
<th>Leukocytes (x10^3)</th>
<th>Neutrophils (%)</th>
<th>MNC (%)</th>
<th>T Cells CD3 (%)</th>
<th>NK Cells 5E6 (%)</th>
<th>B Cells CD19 (%)</th>
<th>Progenitor Cells CFU-GM (10^5 cells)</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Balb/c</td>
<td>PB</td>
<td>4</td>
<td>4.9 (3.4-8.2)</td>
<td>10 (10-50)</td>
<td>90</td>
<td>21 (11-32)</td>
<td>7 (4-11)</td>
<td>ND</td>
<td>0.13 (0.06-0.2)</td>
</tr>
<tr>
<td></td>
<td>PBPC</td>
<td>6</td>
<td>59.6 (42.6-73.3)</td>
<td>50 (20-80)</td>
<td>50</td>
<td>32 (15-35)</td>
<td>4 (2-5)</td>
<td>11 (4-13)</td>
<td>0.53 (0.2-0.87)</td>
</tr>
<tr>
<td>DBA/2</td>
<td>PB</td>
<td>6</td>
<td>9.9 (9.4-10.8)</td>
<td>50 (10-60)</td>
<td>50</td>
<td>20 (12-27)</td>
<td>11 (8-13)</td>
<td>ND</td>
<td>0.80 (0.67-3.0)</td>
</tr>
<tr>
<td></td>
<td>PBPC</td>
<td>6</td>
<td>72.9 (51.8-85.8)</td>
<td>40 (15-60)</td>
<td>60</td>
<td>62 (50-71)</td>
<td>5 (2-7)</td>
<td>20 (11-24)</td>
<td>1.13 (0.07-3.6)</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>14 (6-30)</td>
<td>4 (3-4)</td>
<td>ND</td>
<td>4.83 (1.4-11.47)</td>
</tr>
</tbody>
</table>

Characterization of stem cell transplants as administered to leukemia-bearing Balb/c recipients. Medians and ranges are given for data obtained from the pooled grafts derived from 1 or 2 donors. The flow cytometry analysis was performed in parallel in 6 of 10 single transplantation experiments. The percentage of lymphocytes is given as the percentage of nucleated cells. Nucleated cells were also the basis for the calculation of graft size in all experiments. The MoAb 5E6 labels a subpopulation of NK cells. CFU-GM were counted on day +12 after seeding.

Abbreviations: MNC, mononuclear cells; ND, not determined.

after allogeneic MHC-matched PBPC than after syngeneic PBPC. Complete engraftment was achieved with a minimum cell dose of 5 x 10^9 cells/kg BW after syngeneic PBPC; the same cell dose caused a graft failure rate of 19% after allogeneic PBPC. Thus, the immunogenetic difference was another parameter determining engraftment after PBPC. On the basis of fixed numbers of nucleated cells transferred, allogeneic BMT was followed by lower rejection rates (Fig 1) than allogeneic PBPC. The source of hematopoietic stem cells also influenced engraftment kinetics: a cell dose of 5 x 10^7 cells/kg BW gave a substantial slower engraftment after PBPC (>2 leukocytes/mL on day 17) as compared with BMT (>2 leukocytes/mL on day 11). Long-term engraftment was investigated by immunofluorescence staining of recipient splenocytes with an anti-Lyt 1.1 antibody (CD5) and subsequent FACS analysis (Fig 3). Staining of the spleen cells of allo-PBPC recipients 100 days after transplantation showed the majority of T lymphocytes to be of donor origin in all surviving recipients tested (n = 10). High cell numbers of either origin, PBPC and BM, uniformly resulted in complete and very fast engraftment (>2 leukocytes/mL on day 7, Fig 2).

GVHD. The transplantation of 1 x 10^9 cells/kg BW allogeneic MHC-matched PBPCs resulted in stable engraftment, but 4 of 16 (25%) nonleukemic animals died with signs of GVHD. Histological examination showed GVHD-typical infiltration in the liver and skin in these animals. After allogeneic BMT with 1 x 10^9 cells/kg BW, 2 of 8 animals died on days 43 and 93 with signs of GVHD (Fig 4). This type of mortality was not observed after syngeneic PBPC. After depletion of the CD3^+ cells from allogeneic PBPC transplants, only 1 of 17 animals (6%) died with macroscopic signs of GVHD. One of the animals died during the period of TBI-induced aplasia.

GVL effect. To evaluate the GVL effects after transplanting PBPCs or BM cells, the relapse risk of Balb/c mice bearing the lymphoid leukemia A20 and transplanted with PBPCs or BM cells was assessed. After intravenous injection of 1 x 10^5 A20 cells, all untreated animals died after a median of 28 days. TBI with a dose of 7.5 Gy and subsequent syngeneic PBPC with 1 x 10^6 cells/kg BW 2 days after leukemia cell injection resulted in a prolongation of the time to relapse to a median of 43 days; 20% of the animals remained free from leukemia. Transfer of 1 x 10^6 cells/kg BW allogeneic MHC-matched PBPCs after identical pretreatment of the recipients caused a significantly lower relapse rate with freedom from leukemia at 66% (P = .0045). Freedom from leukemia is 40% (median time to relapse, 58 days) after allogeneic BMT and thus significantly lower than after allogeneic PBPC (P = .032; Fig 5).

DISCUSSION

The animal data presented here address some of the important questions related to transplantation of allogeneic PBPCs. In particular, and in contrast to other murine experiments, our model allows the investigation of the GVL activity of PBPCs as opposed to BM grafts. Results of animal experiments can seldom be directly translated into...
the clinic because of inherent differences between the experimental setting and the clinical situation. In our model, recipients (Balb/c) and donors (DBA/2) of hematopoietic stem cells share the MHC complex but differ with respect to the rest of their genetic background. This situation most closely resembles the transplantation of hematopoietic stem cells between HLA-identical donor/recipient pairs, more so in the matched unrelated than in the HLA-identical sibling setting. There are other limitations of the model pertaining to the composition of both marrow and PBPC grafts in mice and humans and their clinical implications. Although other parameters may be more important with respect to certain experimental or clinical endpoints, we chose to primarily characterize marrow and PBPC grafts by their content of nucleated cells and relate comparisons between BMT and PBPC to fixed numbers of nucleated cells present in either type of graft. This is practical and matches the way how clinical BM and PBPC grafts have been characterized in many instances. 10,12,13 We additionally determined the progenitor cell and lymphocyte content of the grafts to enable a comparison of these parameters and their possible relevance with respect to engraftment potential, graft-versus-host reactivity, and GVL effect observed after BMT or PBPC.

We and others have previously shown that the progenitor cell dose in a graft is the decisive parameter to predict the engraftment potential of a hematopoietic stem cell graft if pretransplantation immunosuppression and the immunoge-
cludes any conclusive answer to the question if the relatively low relapse rates observed after PBPCT so far are the consequence of a more vigorous GVL effect. Reliable clinical data on the GVL effect after transplantation of unmanipulated or T-cell–depleted allogeneic PB stem cells will not be available for years because this will need long-term follow-up of a homogeneous patient population grafted with either PBPCs or BM. The results presented here suggest an advantage of PBPC over BM grafts with respect to their antileukemic activity. What are the possible explanations for this finding? First, there have been reports that priming with G-CSF can alter T-cell phenotype and function.\textsuperscript{31,32} It cannot be excluded that these differences not only affect graft-versus-host reactivity but also GVL activity. Second, it has repeatedly been reported that human PBPC grafts contain 10-fold more T cells and 19- to 25-fold more NK cells as compared with BM grafts.\textsuperscript{11-13} In this experimental system, even with a fixed number of nucleated cells administered, we also transferred more T cells and NK cells with PBPCs than with BM grafts.

Fig 4. Survival of nonleukemic Balb/c mice after TBI only (n = 12), transfer of $1 \times 10^5$ syngeneic PBPCs/kg BW (n = 10), and transfer of the same dose of unmanipulated allogeneic PBPC (n = 16) or CD3-depleted allogeneic PBPCs (n = 16). T-cell depletion was performed by immunomagnetic removal of these cells by anti-CD3–coated magnetic beads, resulting in less than 1% CD3$^+$ cells as shown by flow cytometry.

Fig 5. Freedom from leukemia of Balb/c mice injected with $1 \times 10^5$ cells of the B-lymphocytic leukemia cell line A20. Recipients received no further treatment (n = 10) or received TBI with a dose of 7.5 Gy followed by transplantation of hematopoietic stem cells 2 days after leukemia cell injection. The transplants were either syngeneic PBPCs (n = 22), allogeneic BM cells (n = 23), or allogeneic PBPCs (n = 26).
tive cells but could result in a substantial increase of GVL-reactive cells. Further experiments using the experimental model reported here are underway to further elucidate the relative roles of T cells and NK cells triggering graft-versus-host reactivity and GVL activity. These experiments will hopefully shed further light on the basic biology of graft-versus-host reactivity and GVL effects and give some indication how allogeneic PBPC grafts should best be manipulated to avoid GVHD but preserve their antileukemic potential. The experiments reported here have encouraged us to continue with the clinical evaluation of allogeneic PBPC.

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