Influence of Cell Dose and Graft-Versus-Host Reactivity on Rejection Rates After Allogeneic Bone Marrow Transplantation

By Lutz Uharek, Winfried Gassmann, Bertram Glass, Jörg Steinmann, Helmut Loefller, and Wolfgang Mueller-Ruchholtz

The number of cells transplanted and their capacity to induce graft-versus-host reactivity (GvHR) are two factors that are suspected to influence the engraftment of allogeneic bone marrow. We have investigated their impact on graft rejection rates in busulfan-treated LEW rats. In a series of experiments, we varied (1) the number of marrow cells transferred (1, 5, 10, 20, 30, and 40 $\times 10^7$), (2) the degree of pretransplant immunosuppression (1.5, 3.0, and 4.5 Gy of total body irradiation [TBI]; 0, 30, 60, 90, 120, and 180 mg/kg cyclophosphamide), and (3) the ability of the marrow graft to induce classical GvHR against major histocompatibility complex (MHC) antigens [semiallogeneic (CAP $\times$ LEW)F1 or CAP rats as marrow donors]. Reducing either the immunosuppressive pretreatment or the number of cells transplanted resulted in a stepwise increase in rejection rates. However, every reduction in the size of the marrow inoculum was compensated by increased immunosuppression and vice versa. While 60 mg/kg cyclophosphamide was sufficient to prevent rejections after grafting of 40 $\times 10^7$ cells, 90 mg/kg was necessary to ensure 100% engraftment after transplantation of 20 $\times 10^7$ cells, 120 mg/kg after 10 $\times 10^7$ cells, and 180 mg/kg after 1 $\times 10^7$ cells. Since CAP marrow leads to GvHR-mediated immunosuppression in LEW recipients, in contrast to (CAP $\times$ LEW)F1 marrow, we had supposed that lower cell numbers or cyclophosphamide doses are sufficient to achieve engraftment of CAP marrow. Although severe GvHR was present in all animals receiving escalating doses of CAP cells, the rejection rates were the same as for (CAP $\times$ LEW)F1 marrow. In conclusion, we have demonstrated that there is a sensitive balance between the immunosuppression of the host and the number of marrow cells transferred. We were not able to detect a beneficial effect of classical GvHR against MHC antigens on the engraftment of allogeneic marrow. Thus, our results do not support the hypothesis that the loss of GvHR-mediated immunosuppression is responsible for higher rejection rates following transplantation of T-cell–depleted bone marrow.

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**Materials and Methods**

**Experimental animals.** Female Lewis (LEW) rats (RT11), 10 to 16 weeks of age, were used as bone marrow recipients. CAP (RT11) or (CAP $\times$ LEW)F1 rats, aged 10 to 25 weeks and of either sex, served as bone marrow donors. All animals were obtained from our breeding facilities.

**Busulfan.** Tablets containing 0.5 and 2.0 mg of busulfan were crushed in a mortar and suspended in 3 mL of tap water. Immediately after preparation, a dose of 35 mg/kg was administered orally via a gastric tube. BMT was performed 24 hours after treatment with busulfan. Previous studies in this model have shown
that 35 mg/kg of the drug is lethal, but not sufficiently immunosuppressive to allow engraftment of allogeneic bone marrow.\textsuperscript{13}

**Total body irradiation.** Five rats each were placed in plastic boxes (25 × 25 × 5 cm) and irradiated immediately before injection of the bone marrow (\textsuperscript{60}Co-source, source-cage distance 90 cm, field size 30 × 30 cm, dose rate approximately 1 Gy/min).

**Cyclophosphamide.** Cyclophosphamide was dissolved in distilled water, diluted in normal saline, and injected intraperitoneally on day 2 if the dose did not exceed 60 mg/kg. Higher doses were divided equally and administered on days 3 and 2.

**Bone marrow preparation.** Donor animals were killed by cervical dislocation under ether anesthesia. Marrow was rinsed from femurs, tibias, and humeri with 0.5 mL of normal rat serum. Cells were washed once and resuspended in RPMI 1640. Nucleated cells were counted in a Thoma chamber (Eydam, Kiel, Germany). Immediately after preparation, cells were injected in a constant 2-mL vol into the lateral tail vein of the recipients. The day of marrow grafting was termed day 0.

**Blood counts.** To determine whether the death of an animal was due to graft rejection or to other causes, blood counts were determined for each animal on days 7, 10, 13, 16, 19, 22, 32, 42, 52, 62, 72, and 82 posttransplant. Blood was drawn by puncturing the lateral tail vein. A drop of blood was collected in a microhematocrit tube and a leukocyte pipette. The hematocrit, granulocyte, and platelet counts were determined by routine techniques.

**Skin grafting.** All rats surviving the 100-day observation period received an allogeneic CAP skin graft to establish long-term persistence or late rejection of the transplanted marrow. Full-thickness skin grafts, 10 to 15 mm in diameter, were transferred from the anterior chest wall of the donor to that of the recipient. The transplanted skin was observed for signs of rejection until day 50 after skin grafting. If it was rejected, a second skin graft was transferred to exclude infection or mechanical destruction as the cause of graft failure.

**Chimerism analysis using flow cytometry.** Rats were killed 12 days after BMT. Spleen cells were removed aseptically and pressed through plastic sieves. They were suspended in RPMI 1640 and washed twice. Erythrocytes were selectively lysed. Staining was performed by indirect immunofluorescence, and recorded in a fluorescence-activated cell sorter (FACStar Plus; Becton Dickinson, Erembodegem, Belgium). The first antibody was a mouse anti-RTI\textsuperscript{+} monoclonal antibody specific for donor (MRC OX-27; Serotec, Wiesbaden, Germany), the second antibody was a fluorescein isothiocyanate-conjugated (FITC) anti-mouse IgG Fc (Dianova, Hamburg, Germany). The threshold for positive staining was such that the number of cells stained with the second antibody alone was less than 5%.

**Mixed leukocyte reaction.** Using 96-well microtiter plates, 1 × 10\textsuperscript{6} peripheral blood mononuclear cells (PBMC) from (CAP × LEW)FI rats were cocultured with 2 × 10\textsuperscript{6} irradiated (10 Gy) PBMC of either LEW, DA (RTI\textsuperscript{+}), or (CAP × LEW)FI origin. The culture medium consisted of RPMI 1640 supplemented with 15% fetal calf serum (FCS). On day 4, [\textsuperscript{3}H]-thymidine was added and 24 hours later cells were harvested for counting.

**Definitions.** Death after day 6 with failure to attain a granulocyte count greater than 500/\mu L is considered to be caused by primary rejection of the allogeneic marrow graft or by engraftment failure in case of syngeneic transplantation. Secondary rejection is death in secondary aplasia (granulocyte count < 500/\mu L) after initial engraftment as defined by the aforementioned criterion. Deaths due to other reasons include all deaths occurring in animals not fulfilling the rejection criteria. Deaths occurring before day 7 were considered toxic irrespective of hematological parameters.

**RESULTS**

**Effect of cell dose on the rejection of semiallogeneic bone marrow.** We recently demonstrated that 4.5 Gy of TBI is sufficiently immunosuppressive to ensure engraftment of 40 × 10\textsuperscript{6} (CAP × LEW)FI bone marrow cells in busulfan-treated LEW rats.\textsuperscript{13} To investigate the relationship between cell number and graft survival, the marrow inoculum size was reduced to 30, 20, 10, or 5 × 10\textsuperscript{7} cells. As shown in Fig 1, the rejection rate increased continuously from 0% after injection of 40 × 10\textsuperscript{6} cells to 100% after administration of 5 × 10\textsuperscript{7} cells in pilot experiments. The transplantation of very small cell numbers (5 × 10\textsuperscript{7} cells) was exclusively followed by rejection of the engraftment failure type (primary rejection), while additional secondary rejections were observed within 2 weeks after grafting of higher cell doses (10 to 30 × 10\textsuperscript{7} cells). One late rejection masked by autologous recovery was seen after transplantation of 40 × 10\textsuperscript{7} cells, as indicated by rejection of a CAP skin graft (data not shown). These findings suggested that a relatively small reduction in the size of the marrow inoculum can result in significantly increased rejection rates.

**Relationship between cell dose and pretransplant immunosuppression.** For a systematic investigation of the relationship between rejection rates, marrow cell dose, and pretransplant immunosuppression, cyclophosphamide doses ranging from 30 to 180 mg/kg were administered to LEW rats receiving either 1, 5, 10, 20, or 40 × 10\textsuperscript{7} (CAP × LEW)FI marrow cells. Cyclophosphamide was used instead of TBI, since irradiation doses of greater than 4.5 Gy were associated with higher nonhematological toxicity when administered in conjunction with busulfan. A series of 12 independent experiments was conducted. To exclude any bias due to group effects, six to 12 different experimental conditions (cyclophosphamide/cell dose combinations) were tested in each single experiment.

As can be seen in Fig 2, marrow cell number and cyclophosphamide dose behave as two independent factors.
with regard to their influence on marrow graft rejection rates. After pretreatment with busulfan and 60 mg/kg cyclophosphamide, the frequency of graft rejections decreased steadily from 87% after transplantation of $5 \times 10^7$ cells to 0% after administration of $40 \times 10^7$ cells. These results were comparable to those observed after busulfan plus 4.5 Gy of TBI. At least for the cell doses tested here, the effect of reduced marrow inoculum size could be compensated by higher doses of cyclophosphamide. While 60 mg/kg was sufficient to prevent rejection after grafting of $40 \times 10^7$ cells, 90, 120, or 180 mg/kg was necessary to ensure 100% engraftment after transplantation of 20, 10, or $1 \times 10^7$ cells, respectively.

**Unstable engraftment following low doses of either cyclophosphamide or marrow cells.** After administration of cyclophosphamide doses or cell numbers just below those ensuring 100% engraftment, up to 33% of secondary rejections were observed (Fig 2). This type of rejection is characterized initially by increasing granulocyte and platelet counts, followed by secondary pancytopenia 2 to 3 weeks after transplantation. In this critical dose range, survival of an animal does not necessarily imply persistence of donor hematopoiesis. As shown in Table 1, four animals rejected the donor-type skin graft transplanted on day 100. These rats had either received low cell doses (1 or $5 \times 10^7$) or had been treated with the lowest dose of cyclophosphamide (30 mg/kg).

**Cell dose effects on donor-type chimerism at day 12 after transplantation of semiallogeneic marrow.** Rats were killed on day 12 and the percentage of donor lymphoid and myeloid spleen cells was determined. In these experiments, animals were conditioned with 35 mg/kg busulfan and 60 mg/kg cyclophosphamide before transplantation of 10, 20, or $40 \times 10^7$ bone marrow cells. As shown in Table 2, the percentage of donor-type cells was related to the number of cells grafted: a median of 68% donor cells was observed after transplantation of $4 \times 10^7$ cells, as opposed to only 5% donor cells when $1 \times 10^7$ marrow cells were grafted. These results correspond well with rejection rates defined by death in granulocytopenia (Fig 2), indicating that the absence of peripheral granulocytes is a valid marker of rejection in this model.

**Cell dose effects on the kinetics of hematopoietic reconstitution after transplantation of semiallogeneic marrow.** Bone marrow cell dose and immunosuppressive treatment are crucial factors with respect to the speed of hematological recovery, as demonstrated by blood counts on day 7 posttransplant (Fig 3). Increased marrow cell doses resulted in higher granulocyte, platelet, and hematocrit counts on day 7. Since this effect is not restricted to cell numbers that are critical for marrow engraftment, it does not simply reflect the relationship between cell dose and rejection rate. Although, for example, transplantation of either 5 or

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**Table 1. Long-Term Persistence of Grafted Semiallogeneic (CAP x LEW)F1 Marrow, Using a CAP Skin Graft as a Control**

<table>
<thead>
<tr>
<th>Cyclophosphamide (mg/kg)</th>
<th>Cell Dose Transplanted</th>
<th>Incidence of CAP Skin Graft Rejection</th>
</tr>
</thead>
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<tr>
<td>30</td>
<td>$10 \times 10^7$</td>
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<tr>
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<td>$40 \times 10^7$</td>
<td>0/1</td>
</tr>
<tr>
<td>60</td>
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</tr>
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</tr>
<tr>
<td></td>
<td>$10 \times 10^7$</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Rejection of the CAP skin, grafted at day 100, strongly indicates rejection of the grafted marrow that is masked by autologous recovery.
with busulfan plus 120 mg/kg cyclophosphamide (Fig 2), these two cell doses differed with regard to their effect on the kinetics of hematopoietic reconstitution. After transplantation of 10^10 cells, significantly (P < .05) higher granulocyte (1,600 ± 700/μL) and platelet counts (>100,000 ± 58,000/μL) and slightly higher hematocrit counts (37.6% ± 35.2) were achieved on day 7 posttransplant (Fig 3).

Presumably as a consequence of late hematopoietic recovery, transplant-related mortality due to causes other than graft rejection increased when lower cell doses were transferred. All rats receiving the highest marrow cell dose of 40 × 10^7 cells and greater than 30 mg/kg cyclophosphamide survived the full observation period of 100 days, while up to 25% of the animals receiving lower cell doses died despite evidence of lasting engraftment, apparently due to hemorrhagic or infectious complications (data not shown).

As can be seen in Fig 3, grafting of 40 × 10^7 cells was associated with prompt hematopoietic recovery. Hematopoietic reconstitution following transplantation of graded numbers of syngeneic marrow cells. To determine the impact of cell dose on hematopoietic recovery after transplantation of syngeneic marrow, LEW rats received busulfan plus 60 mg/kg cyclophosphamide, followed by transplantation of 1, 0.5, 0.1, or 0.05 × 10^7 syngeneic cells. Table 3 indicates that 1 × 10^7 cells are sufficient for consistent engraftment. Lower cell doses are associated with an increased risk of death in prolonged pancytopenia (graft failure).

The kinetics of blood cell recovery are shown in Fig 4. As in semiallogeneic grafts, there was a marked cell dose effect on the speed of hematopoietic reconstitution. Both granulocyte and platelet recovery were more rapid when higher cell doses had been administered.

Rejection rates after transplantation of GvHR-reactive fully allogeneic marrow as compared with transplantation of GvHR-nonreactive semiallogeneic grafts. To investigate whether classical GvHR has an engraftment-promoting effect, increasing doses of either GvHR-reactive CAP or GvHR-nonreactive (CAP × LEW)F1 marrow were transferred. In eight independent experiments, LEW rats received 40, 20, 10, or 1 × 10^7 cells of either origin (Fig 5). All of these animals were pretreated with 60 mg/kg cyclophosphamide in addition to 35 mg/kg busulfan. Since only data from parallel experiments were considered for comparison, the rejection rates indicated for semiallogeneic transplantation slightly differ from those shown in Fig 2.

The results shown in Fig 5 clearly demonstrate that there is no marked difference between rejection rates following semiallogeneic and fully allogeneic marrow transplantation. In contrast to our initial assumption, a nonsignificant trend toward higher rejection rates following transplantation of fully allogeneic marrow was observed. These findings are not attributable to weak GvHR in our fully allogeneic donor-recipient combination, since all animals that did not die due to graft rejection subsequently died with classical signs of GvHD.

These results are confirmed by another set of experiments (Fig 6). LEW rats received increasing doses of TBI (1.5, 3, or 4.5 Gy) before transplantation of a constant number (40 × 10^7) of either CAP or (CAP × LEW)F1 marrow cells. Although these experiments were not based on a controlled design, the respective rejection rates were similar.

Donor-type chimerism after transplantation of GvHR-reactive fully allogeneic marrow. Since GvHR might have caused granulocytopenia and death without rejection, spleen cells of CAP marrow recipients were tested for the presence of donor-type MHC antigens at day 12 after transplantation. LEW rats received increasing doses of CAP marrow (40, 20, or 10 × 10^7 cells) following pretreatment with 60 mg/kg cyclophosphamide given in addition to 35 mg/kg busulfan (Table 2). As in semiallogeneic grafts, there was a clear relationship between cell dose and donor-type chimerism determined on day 12 posttransplantation: a median of 78% donor cells after 40 × 10^7 cells contrasted with 1% after 10 × 10^7 cells. Even after 4 × 10^7 cells were grafted, there were absolutely no donor cells detectable when no immunosuppression was administered in addition to the myeloablative dose of busulfan (n = 4, data not shown). Again, we observed no significant difference between GvHR-nonreactive (CAP × LEW)F1 and GvHR-reactive CAP marrow grafts, as indicated by almost identical rates of donor-type spleen cells for every marrow cell dose tested. Thus, we were not able to find any evidence that GvHR directed against MHC antigens has an engraftment-promoting effect.

In vitro tests of T-cell-mediated GvHR. For genetic reasons, classical anti-host-directed alloreactivity should be absent when F1 marrow is grafted into parental animals.
We have previously demonstrated that the busulfan-treated rat can be used for comparing the immunosuppressive effectiveness of cytostatic agents administered before allogeneic BMT. The experiments reported here were undertaken to elucidate the impact of cell dose and of GvHR-induced immunosuppression on rejection rates in this experimental system.

Early studies in different animal models showed that the prerequisites for successful engraftment of allogeneic stem cells are adequate marrow cell dose and sufficient immunosuppression of the host. To investigate the relationship between cell dose and immunosuppression more systematically, busulfan-treated LEW rats additionally received increasing doses of cyclophosphamide (30, 60, 90, 120, or 180 mg/kg) before transplantation of graded numbers (1, 5, 10, 20, or $40 \times 10^7$) of (CAP x LEW)F1 marrow cells. Our data showed a sensitive balance between engraftment and the amount of marrow transplanted on the one side, and the immunosuppressive efficacy of the conditioning regimen on the other (Fig 2). The size of the graft clearly determined the immunosuppression necessary to allow its take. Whereas $1 \times 10^7$ cells required 180 mg/kg of cyclophosphamide to ensure their engraftment, 60 mg/kg of the drug was sufficient to allow allogeneic repopulation following transplantation of $40 \times 10^7$ cells. In other words, the deleterious effect of lowering the cell dose could be compensated by increasing the dose of cyclophosphamide and vice versa.

A number of previous animal studies have indicated that the size of the marrow inoculum plays a decisive role in the engraftment of syngeneic or allogeneic bone marrow. In busulfan-treated rats, Tutschka and Santos have already demonstrated that the cell dose plays a crucial role in engraftment of fully allogeneic marrow. However, systematic titration studies with threshold numbers of allogeneic cells are rare. Moreover, the data presented here clearly show that the impact of cell dose effects depends on the immunosuppression of the host. Therefore, the precise description of cell dose effects should include information about the relative immunosuppressive activity of the particular conditioning regimen. On the other hand, our data emphasize that the amount of marrow transplanted is a critical factor when conditioning regimens are compared for their engraftment-promoting capacity.

Hematological and immunological mechanisms may be

Since this premise is fundamental for the interpretation of our data, we performed mixed leukocyte cultures to prove that there is virtually no T-cell-mediated GvHR in our system. The results are shown in Table 4. Using (CAP x LEW)F1 lymphocytes as effector cells, we were unable to demonstrate T-cell proliferation in response to LEW target cells, indicating that no T-cell-mediated GvHR of F1 donors against LEW recipients is detectable with this assay.

**Table 3. Graft Failure Rates After Transplantation of Graded Numbers of Syngeneic Marrow Cells.**

<table>
<thead>
<tr>
<th>Cell Dose Transplanted</th>
<th>Graft Failure Rate</th>
<th>Survival Rate (until day 100)</th>
</tr>
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<tbody>
<tr>
<td>$1 \times 10^7$</td>
<td>0/6</td>
<td>6/6</td>
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<td>1/14</td>
<td>13/14</td>
</tr>
<tr>
<td>$5 \times 10^4$</td>
<td>3/12</td>
<td>8/12</td>
</tr>
</tbody>
</table>

LEW recipients were pretreated with 35 mg/kg busulfan plus 60 mg/kg cyclophosphamide. Graft failure was defined as death of an animal with granulocytes less than 500/µL.
CELL DOSE, GVHR, AND MARROW GRAFT REJECTION

A third mechanism, namely the active suppression of lymphohematopoietic cells of the host by alloreactive cells of the graft, is at least of minor importance, since T cells of F1 origin are unable to act against parental MHC antigens. This premise was confirmed in our F1 → P system by the complete absence of GvHD in vivo and by the lack of mixed leukocyte reaction activity in vitro (Table 4). On the other hand, these data do not indicate that there is absolutely no activity of F1 cells against parental hematopoietic cells. In mice, such recognition has been demonstrated, and there is also evidence that a similar phenomenon can occur in rats. It has been proposed that natural killer cells act as effector cells of such antiparental activity known as hybrid resistance. In this context, it would have been of interest whether (LEW × CAP)F1 rats show resistance against CAP as compared with syngeneic marrow. However, in this strain combination, which is characterized by high alloreactivity, such experiments are hampered by overwhelming GvHD leading to death within a few days. Experiments

Fig 4. Influence of cell dose on the kinetics of hematopoietic reconstitution after transplantation of syngeneic bone marrow. LEW recipients were pretreated with 35 mg/kg busulfan plus 60 mg/kg cyclophosphamide. The number of animals per data point is equivalent to that shown in Table 3. Analysis of variance showed that the effects of cell dose and pretransplant immunosuppression on granulocyte and platelet counts are statistically significant (P < .01, P < .05, respectively).

responsible for the effectiveness of higher cell doses in overcoming graft rejection in our model. After allogeneic transplantation, hematopoietic recovery represents the end result of competing donor stem cell proliferation and antidonor activity of the host. Increasing the number of grafted stem cells directly promotes allogeneic hematopoiesis and thus may result in tolerance induction when a critical level of donor-type cells is reached. In addition, increased numbers of allogeneic target cells might prevent the destruction of grafted stem cells by blocking the binding sites of host effector cells, a mechanism known as “cold target inhibition.”

Fig 5. Rejection rates after transplantation of increasing doses of either GvHR-reactive fully allogeneic (CAP) or GvHR-nonreactive semiallogeneic [(CAP × LEW)F1] marrow cells. LEW recipients were pretreated with a myeloablative dose of busulfan (35 mg/kg orally) plus 60 mg/kg cyclophosphamide. The data were pooled from five separate experiments. (■) Secondary or (▲) primary rejection.
using different lymphocyte depletion techniques would help to elucidate whether Fl cells other than alloreactive T cells can exert relevant engraftment-promoting activity by acting against lymphohematopoietic tissue of the parental host. Unfortunately, elaborate purging techniques using monoclonal antibodies are not established in rats, so that we have performed these experiments in a similar mice model. Nevertheless, based on the in vivo and in vitro data presented here, we conclude that the effectiveness of higher cell doses in overcoming graft rejection does not depend on classical T-cell-mediated GVHR against MHC antigens.

Our results are in agreement with clinical observations suggesting that marrow graft rejection is not an all-or-nothing phenomenon. It represents a continuum with at least three rejection types that can be distinguished: (1) rapid rejection without evidence of donor-type hematopoiesis, (2) delayed rejection resulting in secondary pancytopenia, and (3) late rejection masked by autologous recovery. For the busulfan-treated rat, we have demonstrated that the outcome can easily be assessed with the help of hematological parameters and donor-type skin grafts. As shown in Table 2, comparable results were obtained when markers of donor cell engraftment were used to investigate graft survival. We have shown that incidence and type of rejection are determined by marrow cell dose and immunosuppressive treatment of the host. Moreover, even in cases of verified long-term engraftment, the regeneration of peripheral blood neutrophil and platelet counts was shown to depend on these two factors (Fig 3). A similar correlation between marrow cell dose and speed of engraftment has been described for autologous or syngeneic transplantation in a number of other animal models. We have confirmed these observations (Fig 4).

In the clinical context, it is difficult to examine the relationship between cell dose and engraftment, since a variety of other factors, including presensitization, histoincompatibility, and posttransplant immunosuppression, influence the individual pattern of hematological recovery. In patients transplanted for severe aplastic anemia (SAA), improved recovery of neutrophil counts and increased engraftment rates after infusion of high marrow cell doses have been reported. However, in recipients of unmanipulated HLA-matched bone marrow transplanted for hematological malignancies, no positive correlation could be detected between the number of nucleated cells infused and time until engraftment. According to the data presented here, the most pronounced cell dose effects should be expected when the immunosuppressive pretreatment is not sufficient to allow 100% engraftment, as is the case in patients transplanted for SAA and in recipients of HLA-mismatched or Fl-cell-depleted marrow grafts. Recent data from these patient groups have confirmed this assumption.

Since persisting host lymphocytes are among the targets of graft-derived T cells, it has been postulated that a strong GVHR would prevent marrow graft rejection in case of suboptimal pretreatment of the host. Semiallogeneic Fl bone marrow genetically lacks the capacity to mount a classical GVH response against parental cells, and thus increased rejection rates should be the consequence. We failed to detect such an effect. In a first attempt to demonstrate the engraftment-promoting effectiveness of GVHR-mediated immunosuppression, LEW rats were treated with different doses of TBI before infusion of either 40 x 10^7 (CAP x LEW)F1 or CAP marrow cells (Fig 6). No differences were observed between the rejection rates in GVHR-reactive grafts and those in GVHR-nonreactive grafts. In a second set of strictly parallel experiments, the animals received busulfan plus 60 mg/kg cyclophosphamide, followed by infusion of increasing numbers of (CAP x LEW)F1 or CAP bone marrow cells (Fig 5). Again, we

![Graph](image-url)
observed nearly the same rejection rates with (CAP × LEW)F1 marrow as with fully allogeneic grafts. It can be argued that GvHR might have caused granulocytopenia and concomitant death without rejection (ie, mucosal injury), thereby resulting in inaccurately high "rejection" rates as defined by our rejection criteria. Therefore, we additionally analyzed the percentage of donor-type lymphoid and myeloid cells at day 12 after grafting of either GvHR-nonreactive (CAP × LEW)F1 or GvHR-reactive CAP marrow. Using this criterion, the results were exactly the same: nearly identical engraftment rates for every marrow cell dose tested (Table 2). These findings are in agreement with a recent report indicating that F1 marrow is just as effective as fully allogeneic marrow for establishing lymphoid chimerism and skin graft tolerance in sublethally irradiated mice.44

In clinical BMT, the high incidence of graft failures following transplantation of T-cell-depleted marrow was taken as evidence for the hypothesis that GvH-mediated immunosuppression would facilitate marrow engraftment. Our experimental data did not support this hypothesis. On the other hand, we cannot exclude the possibility that GvHR plays a limited role in successful engraftment of allogeneic marrow. It may be that the postulated disadvantage of semiallogeneic cells, ie, their inability to induce GvHR, was outweighed by a possible reduction in immunogenicity. Nevertheless, it is evident from the data presented here that the engraftment-promoting effect of GvHR, if it exists, must be lower than or equal to that of reduced immunogenicity.

At least two alternative hypotheses, both of which conform to our data, have been proposed to explain the increased frequency of graft failure following transplantation of T-cell-depleted marrow. First, activated T cells are known to produce hematostimulatory cytokines45 that might be important for successful stem cell engraftment in allogeneic hosts.46 If this hypothesis is correct, higher rejection rates are to be expected after transplantation of T-cell-depleted marrow compared with unmanipulated marrow, regardless of whether GvHR-reactive or GvHR-nonreactive marrow cells are transplanted. Because monoclonal antibodies for the elimination of T cells and T-cell subsets are not available in rats, we have started to investigate this question in lethally irradiated mice.47 Similar to the experiments reported here, the mice received increasing doses of either T-cell–depleted or unmanipulated marrow cells from semi-allogeneic or fully allogeneic donors. In contrast to other investigators, we adjusted the final marrow cell dose for unspecific cell loss due to in vitro manipulations. The data provided no evidence of an increased rejection rate after transplantation of T-cell–depleted semiallogeneic or allogeneic marrow. These results argue against engraftment-promoting effects of Thy-1+ T lymphocytes, regardless of whether GvHR is involved or not.

Second, the in vitro treatment of the marrow might reduce the number of hematopoietic progenitor cells necessary for long-term engraftment. Although T-cell depletion procedures reduce the number of nucleated cells to a considerable degree (ranging from ~60%48 to 90%49), a deleterious effect on the capacity for hematopoietic reconstitution was not detected by in vitro assays.46,49 In addition, data obtained in monkeys have not provided any evidence that purging with monoclonal anti-T-cell antibodies has a negative influence on the repopulating capacity of autologous bone marrow grafts.31 On the other hand, it might be difficult to uncover a moderate reduction in the repopulation capacity of manipulated marrow grafts using autologous or syngeneic control experiments.52

Even though there is no direct evidence that a reduction in the number of hematopoietic progenitor cells contributes to the increased incidence of graft failure after T-cell depletion in clinical BMT, the results reported here should remind us that cell dose variations can exert considerable influence on engraftment rates. Of course, it is not possible to transfer our results directly to the clinical setting. Mainly due to the technique of marrow harvesting, the contamination with peripheral blood leukocytes is higher in man than in exsanguinated rats. Therefore, the number of nucleated cells transplanted per kilogram of body weight is not exactly comparable. Moreover, apart from the problems inherent to interspecies analogies, the conditioning used in our model differs from that conventionally used in clinical BMT.

With these limitations in mind, it may still be of interest to relate our findings to data obtained in clinical BMT. The marrow cell dose usually transferred in the clinical setting is 2 to 4 × 10^8.5 Supposing that the absolute numbers of 1, 5, 10, 20, and 40 × 10^7 rat marrow cells are nearly equivalent to 0.4, 2, 4, 8, and 16 × 10^6 cells/kg, a marrow cell number of 5 to 10 × 10^7 per rat would correspond to the conventional clinical cell dose. In our MHC-mismatched system, 120 mg/kg cyclophosphamide administered in addition to a lethal dose of busulfan is necessary for ensuring engraftment of this cell dose (Fig 2). Lowering either the cyclophosphamide dose to 90 mg/kg or the marrow cell dose to 0.4 × 10^6 cells/kg increased the rejection rate to 47% or 83%, respectively. Therefore, our results would suggest that a 50% to 80% reduction of the standard marrow cell dose can decrease the engraftment rate substantially. As a consequence, manipulations of the graft that are supposed to reduce the content of hematopoietic cells to such a degree should be avoided unless intensified immunosuppression is provided. On the other hand, our data suggest that a 25% reduction of the conventional immunosuppressive treatment could be compensated for by using two or four times as many marrow cells.

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Influence of cell dose and graft-versus-host reactivity on rejection rates after allogeneic bone marrow transplantation

L Uharek, W Gassmann, B Glass, J Steinmann, H Loeffler and W Mueller-Ruchholtz