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 × 10^8), (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 × 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 × 10^8 cells, 90 mg/kg was necessary to ensure 100% engraftment after transplantation of 20 × 10^8 cells, 120 mg/kg after 10 × 10^8 cells, and 180 mg/kg after 1 × 10^8 cells. Since CAP marrow leads to GvHR-mediated immunosuppression in LEW recipients, in contrast to (CAP × 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 × 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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A LLOGENEIC bone marrow transplantation (BMT) has offered new therapeutic prospects for the treatment of hematological malignancies. In the past two decades, most patients have been conditioned with total body irradiation (TBI) and cyclophosphamide and have received unmanipulated HLA-identical marrow. Graft-versus-host disease (GVHD) and leukemic relapse have been the major causes of treatment failure, while graft rejection has occurred only as a rare complication.

When the standard treatment of the graft or of the host is modified to improve GVHD prophylaxis or prevent relapse, graft rejections can emerge as a serious problem. The best known example is the use of T-cell-depleted bone marrow, which minimizes the risk of severe GVHD, even after major histocompatibility complex (MHC)-mismatched transplantation, but is associated with a high incidence of graft rejection. New strategies for marrow pretreatment, making use of recombinant cytokines, might result in similar problems. Modifications of the standard conditioning regimen that are supposed to provide superior antileukemic activity do not always assure sufficient immunosuppression to promote engraftment.

Previously, we described an experimental system for the investigation of graft rejection after allogeneic BMT. Its rationale relies on the fact that lethal doses of busulfan are myeloablative, but not sufficiently immunosuppressive to prevent the rejection of allogeneic bone marrow. In recent studies, we have used this model for comparing the engraftment-promoting effectiveness of new conditioning agents and of different posttransplant immunosuppressive protocols.

Now we have begun a series of experiments to investigate the engraftment-promoting effects of the transplant itself. First, we wanted to determine the impact of the marrow inoculum size on engraftment. Since cell dose effects were expected to depend on the degree of residual alloreactivity directed against the donor, we varied both the size of the graft and the immunosuppressive pretreatment of the host. Second, we asked whether there is an engraftment-promoting effect of GVHR that is mediated by classical alloreactivity directed against MHC antigens on persisting immunocompetent host cells. Since such graft-versus-host reactivity (GVHR)-mediated immunosuppression is absent after infusion of semiallogeneic marrow into parental recipients, increased rejection rates have been expected in cases of GVHR-free transplantation. We therefore compared the rejection rates following transplantation of graded numbers of either GVHR-reactive fully allogeneic marrow cells or GVHR-nonreactive semiallogeneic cells.

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 × 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.

**Total body irradiation.** Five rats each were placed in plastic boxes (25 × 25 × 5 cm) and irradiated immediately before injection of the bone marrow (500-Gray-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).

**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. If it was rejected, a second skin graft was performed by indirect immunofluorescence, and recorded in a hematocrit 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-RTI1 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 secondary antibody alone was less than 5%.

**Mixed leukocyte reaction.** Using 96-well microtiter plates, 1 × 10⁷ peripheral blood mononuclear cells (PBMC) from (CAP × LEW)F1 rats were cocultured with 2 × 10⁷ irradiated (10 Gy) PBMC of either LEW, DA (RT1n), or (CAP × LEW)F1 origin. The culture medium consisted of RPMI 1640 supplemented with 15% fetal calf serum (FCS). On day 4, [3H]-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/μL 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/μL) 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 × 10⁷ (CAP × LEW)F1 bone marrow cells in busulfan-treated LEW rats. To investigate the relationship between cell number and graft survival, the marrow inoculum size was reduced to 30, 20, 10, or 5 × 10⁷ cells. As shown in Fig 1, the rejection rate increased continuously from 0% after injection of 40 × 10⁷ cells to 100% after administration of 5 × 10⁷ cells in pilot experiments. The transplantation of very small cell numbers (5 × 10⁷ 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 × 10⁷ cells). One late rejection masked by autologous recovery was seen after transplantation of 40 × 10⁷ 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 × 10⁷ (CAP × LEW)F1 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 affecting the rejection rate.
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

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>
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<tr>
<td>30</td>
<td>$10 \times 10^7$</td>
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<tr>
<td></td>
<td>$40 \times 10^7$</td>
<td>0/1</td>
</tr>
<tr>
<td>60</td>
<td>$5 \times 10^7$</td>
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</tr>
<tr>
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<td>$10 \times 10^7$</td>
<td>0/19</td>
</tr>
<tr>
<td></td>
<td>$30 \times 10^7$</td>
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<td>$1 \times 10^7$</td>
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</tr>
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<td>0/5</td>
</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.
graded numbers

CELL DOSE, GvHR, AND MARROW GRAFT REJECTION

Statistical significance was established according to the Mann-Whitney two-sample (nonmatched) test. The median percentage of donor-type cells in these two cell doses differed with regard to their effect on recovery, transplant-related mortality due to causes other than classical GvHR has an engraftment-promoting effect, 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 × 10⁷) 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 × 10⁷ 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 × 10⁷ cells contrasted with 1% after 10 × 10⁷ cells. Even after 4 × 10⁷ 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.
DISCUSSION

We have previously demonstrated that the busulfan-treated rat can be used for comparing the immunosuppressive effectiveness of cytostatic agents administered before allogeneic BMT.13,15 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.17-19 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 x 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 x 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 x 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 syngeneic20,21 or allogeneic14,19,22-26 bone marrow. In busulfan-treated rats, Tutschka and Santos27 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.28,29

Hematological and immunological mechanisms may be

| Table 3. Graft Failure Rates After Transplantation of Graded Numbers of Syngeneic Marrow Cells. |
|-------------------------------|--------------|------------------|
| Cell Dose | Graft Failure Rate | Survival Rate (until day 100) |
| Transplanted |                 |                               |
| 1 x 10^7 | 0/6 | 6/6 |
| 5 x 10^6 | 1/9 | 8/9 |
| 1 x 10^8 | 1/14 | 13/14 |
| 5 x 10^8 | 3/12 | 8/12 |

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.

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.

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Fig 3. Influence of cell dose and pretransplant immunosuppression on peripheral blood cell counts determined in LEW rats 1 week after transplantation of semiallogeneic (CAP x LEW)F1 bone marrow cells. Bone marrow recipients were pretreated with a myeloablative dose of busulfan (35 mg/kg orally) plus increasing doses of cyclophosphamide. The number of animals per data point is equivalent to that shown in Fig 2. Analysis of variance showed that the effects of cell dose and pretransplant immunosuppression on granulocyte and platelet counts are statistically significant (P < 0.01, P < 0.05, respectively).
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.\textsuperscript{33,34} and there is also evidence that a similar phenomenon can occur in rats.\textsuperscript{35} It has been proposed that natural killer cells act as effector cells of such antiparental activity known as hybrid resistance.\textsuperscript{33} In this context, it would have been of interest whether (LEW × CAP)\textsuperscript{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

![Diagram of cell dose, GvHR, and marrow graft rejection](image-url)

**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).

![Diagram of rejection rates after transplantation](image-url)

**Fig 5.** Rejection rates after transplantation of increasing doses of either GvHR-reactive fully allogeneic (CAP) or GvHR-nonreactive semiallogeneic [(CAP × LEW)\textsuperscript{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 F1 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 hematopenia, (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, 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.

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 T-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 F1 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 × 10^5 (CAP × 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 × LEW)F1 or CAP bone marrow cells (Fig 5). Again, we

**Table 4. Missing T-Cell Reactivity of (CAP × LEW)F1 Effector Cells Against LEW Leukocytes in Mixed Leukocyte Culture**

<table>
<thead>
<tr>
<th>Effector Cells</th>
<th>Stimulator Cells</th>
<th>MHC</th>
<th>Experiment</th>
<th>cpm</th>
<th>Stimulation Index</th>
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<tbody>
<tr>
<td>(CAP × LEW)F1</td>
<td>(CAP × LEW)F1</td>
<td>RT1</td>
<td>1</td>
<td>1094</td>
<td>-</td>
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<tr>
<td>(CAP × LEW)F1</td>
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<td>RT1</td>
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</table>

PBMC from (CAP × LEW)F1 rats were cocultured with irradiated PBMC from LEW rats. Irradiated PBMC of DA rats served as positive control, and irradiated syngeneic (CAP × LEW)F1 cells as negative control. On day 4, 1H-thymidine was added and 24 hours later cells were harvested for counting. Statistical significance was established according to the Mann-Whitney two-sample (nonmatched) test. The median percentage of donor-type cells differed significantly (P < .05) between the following groups: (CAP × LEW)F1 v DA; CAP v DA. All other group differences were not significant.
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 cytokines that might be important for successful stem cell engraftment in allogeneic hosts.45 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 grafted. 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 semiallogeneic or fully allogeneic donors. In contrast to other investigators, we adjusted the final marrow cell dose for unsettled cell loss due to in vivo 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%46 to 90%), a deleterious effect on the capacity for hematopoietic reconstruction was not detected by in vitro assays.47,50 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 repopulating capacity of manipulated marrow grafts using autologous or syngeneic control experiments.51

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 × 10⁶.3 Supposing that the absolute numbers of 1, 5, 10, 20, and 40 × 10⁷ rat marrow cells are nearly equivalent to 0.4, 2, 4, 8, and 16 × 10⁶ cells/kg, a marrow cell number of 5 to 10 × 10⁷ 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 × 10⁶ 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 immunsuppressive treatment could be compensated for by using two or four times as many marrow cells.

REFERENCES

3. Thomas ED, Buckner CD, Banaji M, Clift RA, Fefer A,


27. Tutschka PJ, Santos GW: Bone marrow transplantation in the busulfan treated rat III. Relationship between myelosuppression and immunosuppression for conditioning bone marrow recipients. Transplantation 24:52, 1977


44. Pierce GE: Allogeneic versus semiallogeneic F1 bone marrow transplantation into sublethally irradiated MHC-disparate hosts: Effects on mixed lymphoid chimerism, skin graft tolerance, host survival, and alloreactivity. Transplantation 49:138, 1990
47. Uharek L, Glass B, Gassmann W, Loeffler H, Mueller-Ruchholtz W: The role of T cells in bone marrow engraftment. Comparison of rejection rates and long-term chimerism after transplantation of T cell depleted or unmanipulated murine marrow genetically lacking the capacity to induce a GvHR. (manuscript in preparation)
Influence of cell dose and graft-versus-host reactivity on rejection rates after allogeneic bone marrow transplantation

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