Prevention of Lethal Murine Graft Versus Host Disease by Treatment of Donor Cells with L-Leucyl-L-Leucine Methyl Ester

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Abstract

Graft vs. host disease (GVHD) remains one of the main problems associated with bone marrow transplantation. The current studies were undertaken to determine whether treatment of the donor inoculum with the anticytotoxic cell compound L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) would alter the development of GVHD in a murine model. Irradiated recipient mice transplanted with a mixture of control bone marrow and spleen cells from naive semiallogeneic donors died rapidly from GVHD, whereas the recipients of cells incubated with 250 μM Leu-Leu-OMe all survived. In addition, Leu-Leu-OMe treatment of cells obtained from donors immunized against host alloantigens resulted in significantly prolonged survival. Phenotypic characterization of spleen cells from the various groups of mice that had received Leu-Leu-OMe–treated cells and survived consistently revealed the donor phenotype. Treatment of marrow cells with 250 μM Leu-Leu-OMe appeared to have no adverse effects on stem cell function. Erythropoiesis was undiminished, as assayed by splenic 5-iodo-2′-deoxyuridine-^{125}I uptake. Moreover, granulocytic and megakaryocytic regeneration were histologically equivalent in the spleens of recipients of control or Leu-Leu-OMe–treated cells. Treatment of the donor inoculum with Leu-Leu-OMe thus prevents GVHD in this murine strain combination with no apparent stem cell toxicity.

Introduction

Graft vs. host disease (GVHD)[1] remains one of the major problems preventing an expanded use of human bone marrow transplantation (1, 2). Although it is theoretically possible to avoid GVHD by careful histocompatibility matching, it is not currently feasible to match donor and recipient routinely for all major histocompatibility antigens. Moreover, there are no in vitro tests to detect minor antigenic disparities that can also stimulate GVHD (3). Because numerous studies in laboratory animals have shown that removal of immunocompetent T lymphocytes from the donor inoculum will prevent GVHD (4, 5), the approaches to obviate GVHD in man have also focused on deleting T cells from the donor marrow. The available methodology for human T cell depletion, however, has not proven uniformly effective in preventing GVHD (6–9).

In this study we explored a new approach to the prevention of GVHD. Using a murine model of bone marrow transplantation, we examined the effects of treating the donor inoculum with L-leucyl-L-leucine methyl ester (Leu-Leu-OMe), a compound that eliminates cells with cytotoxic potential (10). Leu-Leu-OMe has been shown to be toxic to human natural killer cells (NK), activated cytotoxic T cells (CTL), precursors of CTL (pre-CTL), and monocytes (11). Of importance, both CD8-positive and CD4-positive precursors and effectors of CTL are removed from mixed cell populations by Leu-Leu-OMe. By contrast, helper T cells, B cells, and a variety of nonhemopoietic cells are unaffected (10, 11). In recent experiments murine NK and pre-CTL were found to be very similar to human cells in concentration-dependent sensitivity to Leu-Leu-OMe (12). To determine whether Leu-Leu-OMe might affect the induction of, or alter the pattern of tissue injury in GVHD, we used a murine model of bone marrow transplantation that is skewed toward GVHD in that it crosses major histocompatibility barriers and uses a severalfold excess of donor T cells. The experimental results indicate that Leu-Leu-OMe treatment of the donor inoculum had the capacity to prevent lethal GVHD with no apparent toxic effects on stem cell function.

Methods

Mice. Female, 8–16-wk-old, C57BL/6J (B6) and (C57BL/6J × DBA/2J)F1 mice (B6D2F1) were used as donors and recipients, respectively. For some experiments B6 donors were immunized by injecting 70 × 10^6 nonirradiated B6D2F1 spleen cells intraperitoneally 12 d before use.

Treatment of donor cells and transplantation. Spleen and marrow cells were obtained as previously described (13), and mixed at varying ratios. The mixtures were then washed once and resuspended at a concentration of 2.5 × 10^6 cells/ml either in phosphate-buffered saline (PBS) or in Leu-Leu-OMe dissolved in PBS. Leu-Leu-OMe was synthesized from leucyl-leucine as previously described (10). After a 15-min incubation at room temperature, the cells were washed once, suspended in Hanks' balanced salt solution, counted, and infused via a lateral tail vein into irradiated (950 cGy) F1 recipients.

As previously described, immediately after treatment with Leu-Leu-OMe, there was less than a 5% loss of viable donor cells. Following a 3-h incubation at 37°C, however, the number of Lyt-2$^+$ spleen cells was

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; GVHD, graft vs. host disease; Leu-Leu-OMe, L-leucyl-L-leucine methyl ester; MST, mean survival time; NK, natural killer cells.

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decreased by more than 65%, whereas there was no alteration in the number of L3T4$^+$ cells (12).

Survival studies. Survival times were measured from the day of transplantation to the day of death. Deaths occurring within 8 d of transplantation were considered to be the result of radiation-induced gastroenteritis and were excluded. The nonparametric Mann-Whitney test was used to determine whether the median survival times (MST) differed between groups (14).

Assessment of spleen cell H-2 phenotype. Spleen cells from control mice (normal B6 or B6D2F$_1$) and long-term BMTx survivors (B6 → B6D2F1) were depleted of B cells by panning on goat anti–mouse immunoglobulin-coated petri dishes (15). Aliquots of B cell–depleted spleen cells were incubated either with antibody 30-5-7.S (anti-L^d [16]), antibody 28-18-3.S (anti-k^b [17]), or with a relevant isotype control antibody for 30 min at 4°C. Cells were then washed and incubated with a fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (GAMIG-FITC, Cappell Laboratories, Cochranville, PA) and analyzed on a flow cytometer (Ortho system 50 HH, Ortho Diagnostics, Raritan, NJ).

Measurement of stem cell function. Bone marrow cells or marrow and spleen cell mixtures were incubated in PBS with or without 250 μM Leu-Leu-OMe as above and then infused into 950 cGy irradiated syngeneic recipients. 5 d later, splenic 5-iodo-2′-deoxyuridine-^{125}I (^{125}I-UdR) uptake was measured as previously described (15).

Histology. Organs of recipient mice were fixed in 10% buffered formalin. Sections were stained with Hematoxylin and Eosin.

Results

Survival of transplanted mice. To assess the effects of Leu-Leu-OMe treatment of the donor inoculum on lethal GVHD, we incubated B6 donor cells with varying concentrations of Leu-Leu-OMe before infusing them into irradiated B6D2F1 recipients. As shown in Fig. 1, the recipients of 25 × 10^6 untreated donor cells died rapidly. Recipients given 25 × 10^6 cells preincubated with 125 μM Leu-Leu-OMe had significantly delayed mortality from GVHD (median survival time of 41.5 vs. 19 d, $P < 0.001$), but all eventually died. By contrast, recipients of donor cells treated with 250 μM Leu-Leu-OMe all survived. Similar results were obtained in four separate experiments using 25 × 10^6 donor cells treated with 250 μM Leu-Leu-OMe (data not shown). When the donor inoculum was doubled to 50 × 10^6 cells, all of the recipients of donor inocula treated with 250 μM Leu-Leu-OMe survived. Even when immune donors were used and the control mice experienced accelerated GVHD (Fig. 2), the recipients of donor cells treated with 250 μM Leu-Leu-OMe manifested significantly prolonged survival (median survival times of 49 vs. 11 d, $P < 0.001$) and three of nine became long-term survivors.

GVHD-mediated tissue injury. Control mice from all three experimental conditions exhibited acute cutaneous GVHD within 2 wk of transplantation, manifested clinically by alopecia and scaling, and histologically by epidermal basal layer liquefaction, epidermal lymphocytic infiltration, and a mononuclear cell infiltrate in the dermis. The recipients of 25 × 10^6 donor cells treated with 125 μM Leu-Leu-OMe also manifested evidence of acute GVHD, although at somewhat diminished intensity, whereas the recipients of 25 × 10^6 cells treated with 250 μM Leu-Leu-OMe had no evidence of cutaneous GVHD by inspection or biopsy early after transplantation and no evidence of GVHD in any target organs at the time of sacrifice, 125 d after bone marrow transplantation. By contrast, all five recipients of 50 × 10^6 Leu-Leu-OMe treated cells manifested acute cutaneous GVHD. Two of five developed a progressive widespread dermal sclerosis beginning 3 wk after transplantation. This was most severe 3 mo after transplantation and then substantially resolved. At the time of sacrifice (275 d after bone marrow transplantation) these two mice had only mild residual dermal scle-

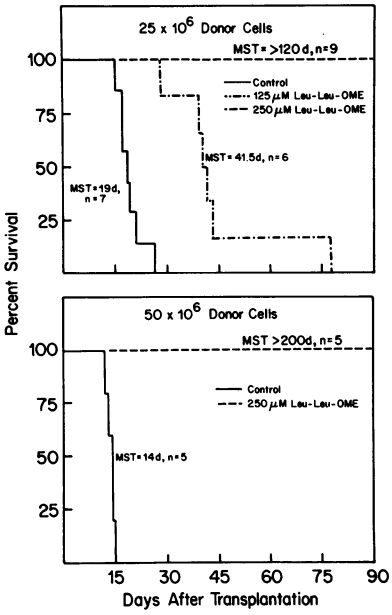


Figure 1. Survival of naive B6 → B6D2F1 mice. Donor bone marrow and spleen cells were obtained from naive B6 mice and mixed at various ratios prior to incubation and transfer into lethally irradiated B6D2F1 mice. (*Top*) When 25 × 10^6 donor cells (spleen to marrow ratio 2.5:1) were transplanted, recipients of untreated cells had a median survival time (MST) of 19 d, recipients of cells treated with 125 μM Leu-Leu-OMe had a MST of 41.5 d ($P < 0.001$), and recipients of cells treated with 250 μM Leu-Leu-OMe all survived >120 d. (*Bottom*) When 50 × 10^6 donor cells (spleen to marrow ratio 5:1) were transplanted, recipients of untreated cells had an MST of 14 d, and recipients of cells treated with 250 μM Leu-Leu-OMe all survived >200 d.

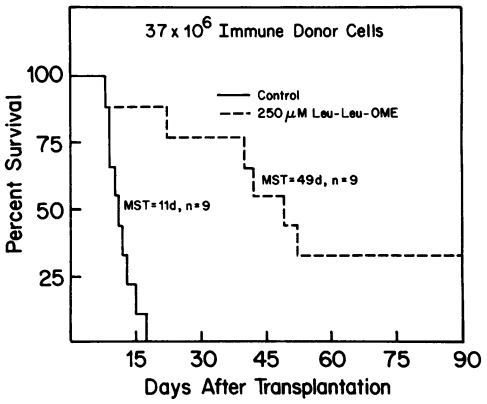


Figure 2. Survival of immune B6 → B6D2F1 mice. Donor bone marrow and spleen cells were obtained from B6 mice previously immunized against B6D2F1 alloantigens, incubated in the presence or absence of Leu-Leu-OMe, and then transferred into lethally irradiated B6D2F1 mice. When 37 × 10^6 donor cells (spleen to marrow ratio 3.7:1) were transplanted, recipients of untreated cells died with an MST of 11 d and recipients of cells treated with 250 μM Leu-Leu-OMe had a MST of 49 d ($P < 0.001$). Three of nine of the mice receiving Leu-Leu-OMe treated cells survived >90 d.

rosis, and a mild to moderate periportal round cell infiltrate in the liver.

Demonstration of stable chimerism in mice transplanted with Leu-Leu-OMe treated cells. To see whether the donor hemopoietic system remained dominant over that of the irradiated recipient, we characterized the H-2 phenotype of spleen cells from the mice used in the survival experiments at various times after transplantation. Spleen cells from normal B6 (donor, H-2^b) and B6D2F_1 (recipient, H-2^b/H-2^d) mice served as controls. Cells from three separate experimental groups analyzed as long as 275 d after transplantation consistently displayed the phenotype and staining pattern of the homozygous donor and not the H-2 heterozygous F1 recipient (>95% reactivity with anti-H-2^b, <1% reactivity with anti-H-2^d).

Lack of stem cell toxicity. To assay the effects of Leu-Leu-OMe on hemopoietic stem cells, we incubated marrow or mixtures of marrow and spleen cells in PBS with or without Leu-Leu-OMe and measured splenic ^{125}I-UdR uptake 5 d after transplantation into irradiated syngeneic recipients (18). Data in Table I indicate that treatment with 250 μM Leu-Leu-OMe had no deleterious effect on this measure of stem cell function. Since human marrow obtained for use in transplantation has variable amounts of peripheral blood contamination, we also studied the stem cell function of a mixture of murine spleen and marrow incubated with Leu-Leu-OMe. Again there was no decrement in ^{125}I-UdR uptake, suggesting that "bystander" stem cells are not damaged when the numerous cytotoxic cells are lysed by Leu-Leu-OMe. Histologic examination of spleens 5 d after irradiation revealed the expected hypocellularity in the nontransplanted control group and equivalent erythrocytic, granulocytic, and megakaryocytic regeneration in the spleens of recipients of treated or nontreated marrow cells (Fig. 3).

Discussion

This study indicates that Leu-Leu-OMe treatment of donor cells is able to prevent lethal GVHD across major histocompatibility barriers in this strain combination with no detectable stem cell toxicity. Since especially severe GVHD occurs when immune donors are used, it is noteworthy that one 15-min incubation of primed donor cells with 250 μM Leu-Leu-OMe nevertheless significantly increased survival (Fig. 2).

Table I. In Vivo Proliferation of Hemopoietic Stem Cells

Experiment	n*	Cells grafted	Treatment of cells	Splenic ^{125}I-UdR uptake (%) geometric mean (95% CL)‡
1	8	3×10^6	Vehicle	0.87 (0.70–1.09)
	8	3×10^6	Leu-Leu-OMe	0.99 (0.78–1.28)
	5	0		0.01
2	11	3×10^6	Vehicle	0.48 (0.31–0.78)
	11	3×10^6	Leu-Leu-OMe	0.45 (0.30–0.67)
	6	0		0.01
3	6	3×10^6	Vehicle	2.72 (2.29–3.21)
	7	3×10^6	Leu-Leu-OMe	3.02 (2.32–3.91)
4	8	4×10^6	Vehicle	1.06 (0.78–1.47)
	6	4×10^6	Leu-Leu-OMe	0.83 (0.46–1.50)
5	6	2.5×10^6	Vehicle	1.28 (0.68–2.39)
	6	2.5×10^6	Leu-Leu-OMe	1.27 (1.10–1.46)

Marrow alone (experiments 1–3), or a mixture of spleen and marrow at a 4:1 ratio (experiments 4 and 5), were incubated either in PBS alone or in 250 μM Leu-Leu-OMe before being transferred into irradiated syngeneic recipients.

* Mice per group.

‡ CL, confidence limits.

Spleen cells from surviving mice, assessed as long as 275 d after transplantation, consistently displayed the donor H-2 type, demonstrating that stable chimerism had been established. In addition, the absolute numbers of B cells, L3T4$^+$ and Lyt2$^+$ T cells, and NK function in the spleens of long-term B6 → B6D2F1 chimeras were equivalent to that of age-matched animals transplanted with syngeneic bone marrow and spleen cells (B6 → B6) (data not shown). Moreover, functional assessment of spleen cells from B6 → B6D2F_1 chimeras indicated that the immune system of these animals had regained the capacity to proliferate and to generate cytotoxic T cells to third-party stimulators (H-2^k), but remained unresponsive to host alloantigens (manuscript in preparation).

Although the mechanism whereby Leu-Leu-OMe prevents GVHD is unknown, it appears to be distinct from that of other regimens. We have previously observed that the administration of anti-asialo GM_1 in vivo prevents lethal GVHD across minor histocompatibility barriers (19), but have not been able to affect GVHD across major-histocompatability barriers with the same dosage schedule (unpublished observation). Using monoclonal antibodies to cell surface antigens, Korngold and Sprent (20) have recently shown that lethal GVHD resulting from a full H-2 mismatch can only be eliminated by removal of T cells with a pan-T cell reagent, and not with antibodies to either L3T4 or Lyt-2 alone. The incubation of human cells in vitro with 250 μM Leu-Leu-OMe selectively depletes those with cytotoxic potential regardless of phenotype (11); murine lymphocytes are similarly affected (12). Indeed, the concentration-dependent capacity of Leu-Leu-OMe to eliminate murine splenic cytotoxic T cells closely parallels its efficacy in reducing the severity of GVHD. The incubation of B6 spleen cells with 100 μM Leu-Leu-OMe results in complete loss of NK function but only partial reduction of the generation of allospecific cytotoxic T cells (12). As anticipated from the results above, incubation of B6 spleen and marrow cells with 125 μM Leu-Leu-OMe delayed, but did not eliminate death from GVHD (Fig. 1 *A*). Treatment of cells with 250 μM Leu-Leu-OMe caused near total ablation of allocytotoxicity (12) and prevented lethal GVHD (Fig. 1, *A* and *B*). The results support the hypothesis that the depletion of cytotoxic T cell precursors from the donor inoculum and the resultant absence of cytotoxicity during the interval between transplantation and hemopoietic reconstitution may have facilitated the development of stable chimerism in this strain combination. Alternatively, it may be that other unrecognized functions of these same Leu-Leu-OMe sensitive cells are important in the development of GVHD.

The histology of the long-term survivors revealed no evidence of GVHD in any target organ in recipients of 25 million treated cells 125 d after transplantation. The mild residual dermal sclerosis 275 d after transplantation in two of five mice receiving 50 million treated cells was not accompanied by epidermal, dermal, or follicular lymphocytic infiltration (data not shown), and therefore appeared to be an inactive process. A periportal round cell infiltrate was observed in the livers of all five of these mice, however, suggesting that they developed a mild, nonscarring form of chronic hepatic GVHD. We speculate that the few cells with cytotoxic potential that remain after a single treatment with 250

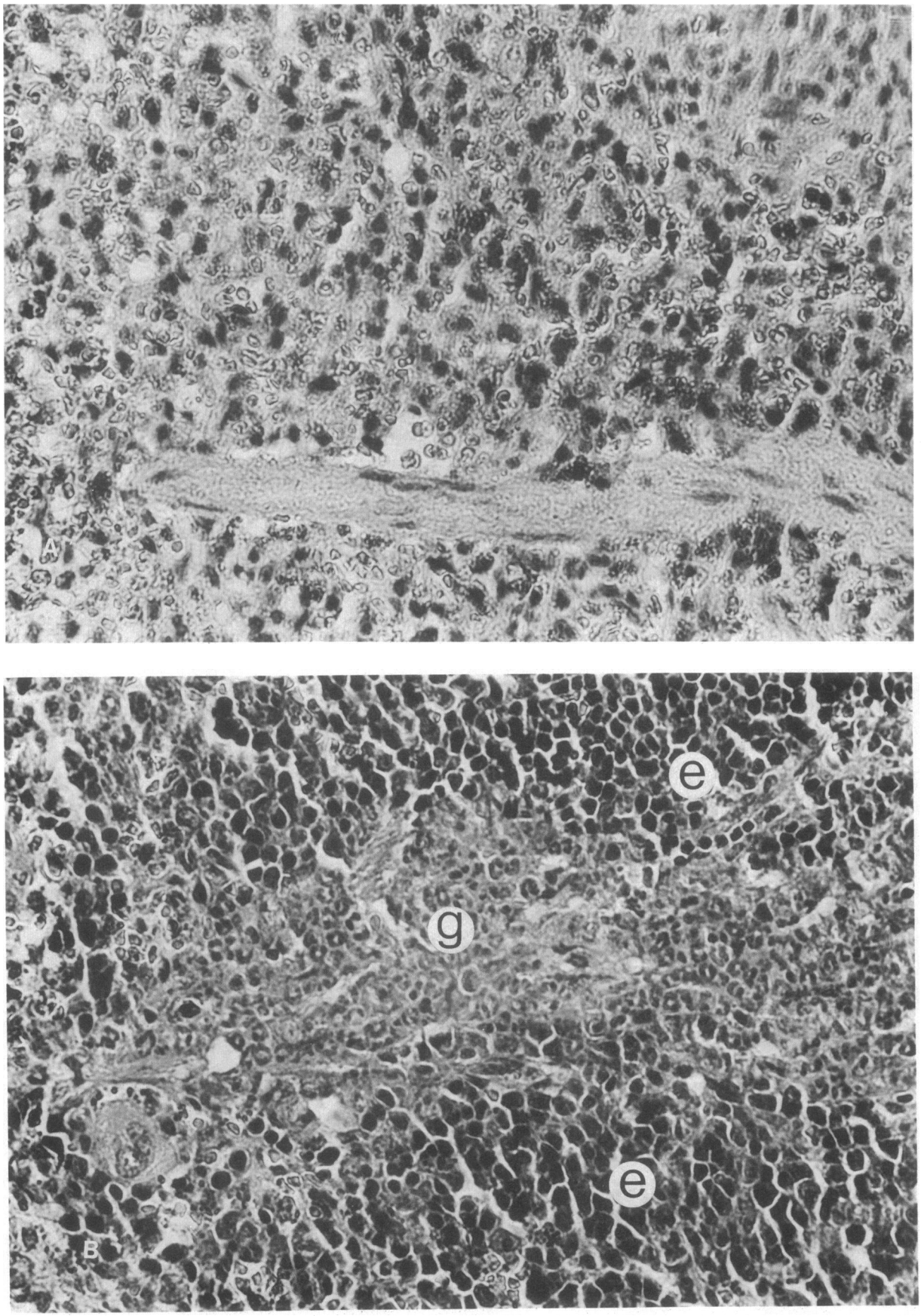
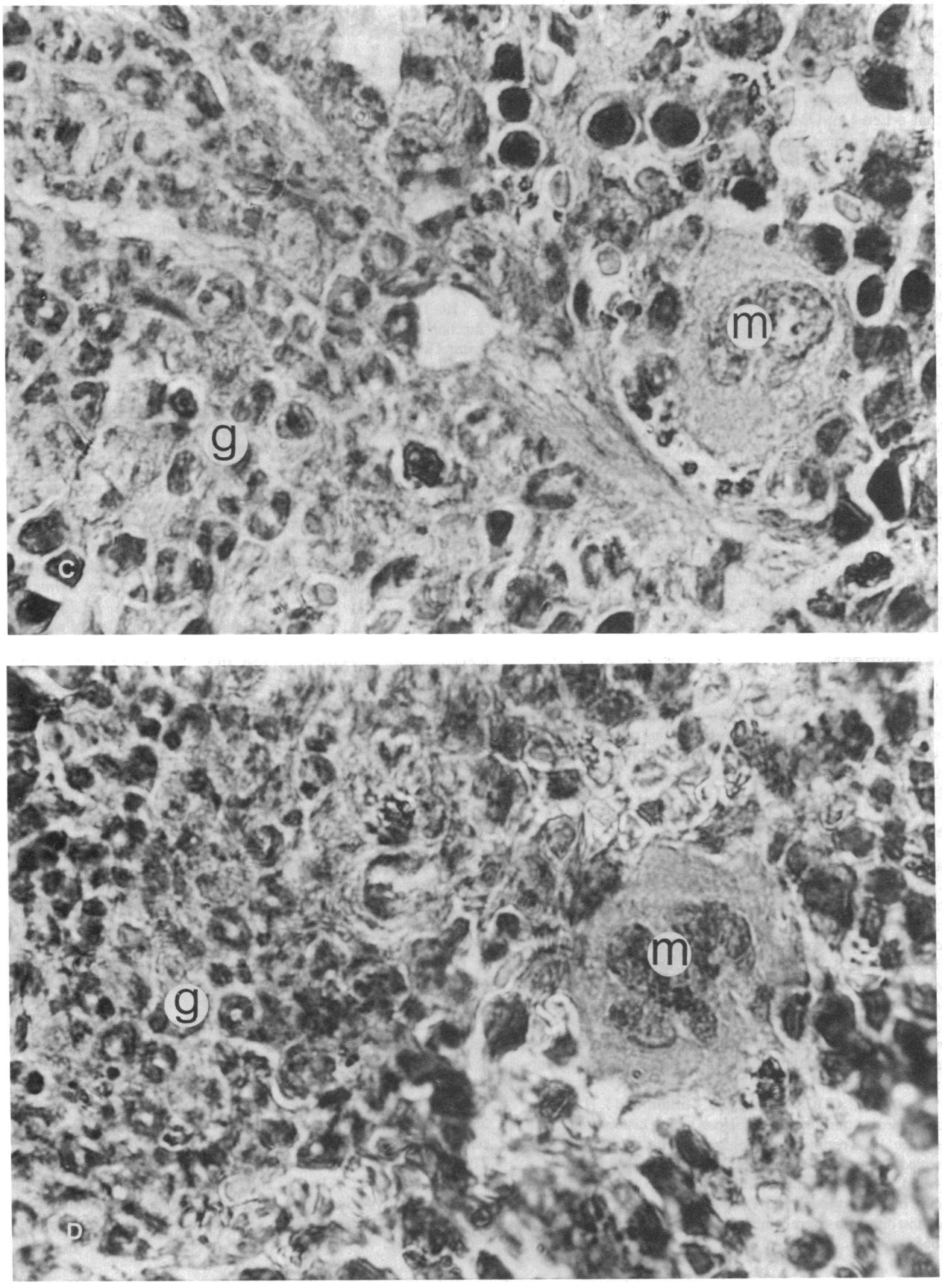


Figure 3. Histology of spleens from irradiated mice. B6 mice were irradiated with 950 cGy and given no marrow (irradiation control), or given syngeneic bone marrow cells that had been incubated in PBS with or without 250 μM Leu-Leu-OMe. 5 d later spleens were examined histologically. (*A*) Irradiation control spleens were sparsely populated, with no hemopoietic elements (× 80). (*B*) Recipients of marrow treated with 250 μM Leu-Leu-OMe had the vast majority of their splenic red pulp packed with erythroblasts (*e*), but also had numerous peritrabecular areas containing



megakaryocytes and granulocytic elements (*g*) (× 80). (*C*) High power view: recipient of marrow treated with 250 μM Leu-Leu-OMe (× 200) with granulocytes (*g*) and megakaryocytes (*m*). (*D*) High power view: recipient of control marrow with granulocytes (*g*) and megakaryocytes (*m*) (× 200). The patterns of repopulation of spleens in (*C*) and (*D*) were identical.

μM Leu-Leu-OMe (<5%) (12) were insufficient in the 25×10^6 inoculum to cause any GVHD, but were sufficient in the 50×10^6 inoculum to cause the self-limited cutaneous GVHD and the mild chronic hepatic GVHD. However, the possibility remains that a Leu-Leu-OMe-resistant, noncytotoxic cell of low frequency in the donor inoculum caused the limited GVHD seen in the recipients of 50×10^6 B6 cells and the more severe GVHD seen in the recipients of immune B6 cells. Studies using other models of murine GVHD have suggested a role for helper T cells in causing a periportal lymphocytic infiltrate in the liver (21) and dermal sclerosis (22). These hypotheses are not mutually exclusive. It should be noted, however, that in the current studies even these nonlethal manifestations of GVHD were only observed when very large numbers of donor Leu-Leu-OMe treated donor cells were used.

The present studies clearly demonstrate the selective nature of the effects of Leu-Leu-OMe. Leu-Leu-OMe had no discernible toxicity for marrow stem cells, yet prevented GVHD. Erythroid regeneration, as assessed by splenic ^{125}I-UdR uptake, was not diminished (Table I) and newly generated granulocytic, erythrocytic and megakaryocytic elements were observed histologically following treatment with Leu-Leu-OMe (Fig. 3). In contrast to current regimens used in human bone marrow transplantation, Leu-Leu-OMe treatment of donor marrow is a relatively rapid and simple technique. Because Leu-Leu-OMe appears to delete certain cells based on their functional capabilities, rather than on their cell surface markers, it may offer a new approach to avoid GVHD in humans.

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