The effects of pretransplant conditioning with high-dose busulfan, a myeloablative but nonimmunosuppressive alkylating agent, on reconstitution of lymphoid tissues by donor cells after bone marrow transplantation (BMT) has not been extensively examined. We used flow cytometric analyses to study the kinetics and extent of lymphocyte repopulation in C57BL/6 mice (immunophenotype Ly-5.2) given graded doses of busulfan (10 to 100 mg/kg) or total body irradiation (TBI; 900 rad) and hematopoietic cell transplantation (HCT; transplantation of bone marrow and spleen cells) from congenic Ly-5.1 donors. Mice transplanted after 10 mg/kg of busulfan had slow and incomplete lymphoid engraftment, only 6% to 11% of lymphocytes in the peripheral blood, lymph nodes, and spleen were positive for Ly-5.1 at 30 days after transplant, slightly increased to 13% to 20% at 60 days, and stabilized at 40% to 46% by 180 days after HCT. Higher doses of busulfan (20 to 100 mg/kg) provided dose-dependent congenic lymphoid reconstitution. Thirty days after HCT, the range of Ly-5.1 cells in blood, lymph nodes, and spleen of Ly-5.2 recipient mice was 43% to 54% after 20 mg/kg of busulfan, 66% to 71% after 50 to 80 mg/kg, and 77% to 85% after 100 mg/kg. Sixty days after transplant, lymphoid chimerism increased to 57% to 68% in 20 mg/kg recipients, 72% to 79% after 35 mg/kg, and 78% to 90% in animals given 50 mg/kg or greater, as seen in radiation chimeras. Despite slower early reconstitution after lower doses of busulfan, donor lymphocytes exceeded 90% to 95% by 120 days after HCT in all mice given at least 20 mg/kg. Even though busulfan lacks directly immunosuppressive properties, virtually complete sustained lymphoid reconstitution by transplanted congenic donor stem cells occurs after its administration. These observations suggest that pretreatment with busulfan may be effective in gene therapy strategies that involve infusion of autologous marrow cells into which functional genes have been inserted.

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tional HCT of single-cell suspensions of bone marrow and spleen cells from congenic Ly-5.1 donors, as previously described; the mean doses (±SD) administered were 28.1 ± 4.0 × 10^6 bone marrow and 69.6 ± 3.9 × 10^6 spleen cells. Cohorts of untreated 10-day-old Ly-5.2 mice were also given HCT with Ly-5.1 cells to determine whether sustained engraftment of congenic cells could occur without pretransplant conditioning. Animals were maintained after HCT in sterilized plastic microisolator cages and given sterilized standard laboratory chow and tap water ad libitum.

Flow cytometric analyses. At selected times after HCT, mice were deeply anesthetized with methoxyflurane and heparinized. Blood samples were obtained by direct cardiac puncture, and the animals were perfused with normal saline to remove residual blood. Blood samples were incubated for 15 minutes at 37°C with erythrocyte lysing buffer, centrifuged for 10 minutes at 1,700 rpm, washed twice, and resuspended in 0.2 g/dL bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Single-cell suspensions of cervical lymph nodes and spleen in Hanks balanced salt solution (HBSS) were centrifuged over Ficoll-diatoxiate (specific gravity 1.083; Sigma, St Louis, MO) for 15 minutes at 3,000 rpm; the erythrocyte-free mononuclear cells at the buffer-Ficoll/diatrizoate interface were washed, centrifuged, and resuspended in BSA-PBS. Aliquots of cell suspensions (1 × 10^6 cells/sample) were incubated on ice for 40 minutes with 100 μL of murine IgG2a anti-Ly-5.1 or anti-Ly-5.2 monoclonal antibody; control samples were incubated with 100 μL of 1.0 g/dL BSA. Cell suspensions were centrifuged for 10 minutes at 1,700 rpm at 5°C, washed twice with BSA-PBS, and then incubated on ice for 40 minutes with 100 μL of 1.50 dilution of fluorescein isothiocyanate (FITC)-labeled heavy-chain-specific goat anti-mouse IgG2a antibody (Southern Biotechnology Associates, Birmingham, AL). Cell samples were recentrifuged and rewashed twice, and resuspended in 1.0 g/dL paraformaldehyde in PBS. Cell analyses were performed on an EPICS Model 752 apparatus (Coulter Diagnostics, Hialeah, FL) equipped with confocal optics and a 90-5 argon laser (Coherent, Palo Alto, CA) tuned to 488 nm (300 mW), and fluorescent emission was collected with a 525 nm bandpass filter. Lymphocytes were gated on 90° and forward-angle light scatter (granularity v size) histograms generated by analysis of 10,000 gated cells for each cell type on each specimen; positive cells were determined by integration of fluorescent histograms in the lymphocyte-gated population. Tissue samples from three mice were assayed at each data point in each experiment.

RESULTS

Mice given transplants of congenic Ly-5.1 bone marrow and spleen cells without pre-HCT conditioning did not have sustained engraftment of donor lymphoid cells, although low levels of Ly-5.1+ lymphocytes were detected for several months after HCT. Thirty days after HCT, 14% of lymphocytes in the peripheral blood and approximately 10% in lymph nodes and spleen were Ly-5.1+, and increased to 21% in blood and 9% to 14% in lymph nodes and spleen by 60 days after HCT. Ninety to 120 days after transplant, donor lymphocytes declined to background levels seen in untransplanted Ly-5.2 controls (Fig 1).

The rapidity and extent of donor Ly-5.1 lymphoid engraftment after congenic HCT in Ly-5.2 recipients was correlated with busulfan dose. In mice transplanted after 10 mg/kg of busulfan, flow cytometry demonstrated that only 8% to 14% of lymphocytes in the peripheral blood, lymph nodes, and spleen were of donor origin 30 days after HCT and slightly increased to 13% to 20% by 60 days after transplant, similar to values obtained in animals given HCT without busulfan (Fig 1). In contrast to untreated recipients of congenic bone marrow and spleen cells, animals transplanted after 10 mg/kg of busulfan had sustained, albeit partial, engraftment of donor Ly-5.1 lymphocytes. Ninety days after HCT, mice pretreated with this low dose of busulfan had 51% donor lymphocytes in peripheral blood, and 38% in lymph nodes and spleen. As late as 180 days after HCT following 10 mg/kg busulfan, 46% of lymphocytes in peripheral blood, 43% in lymph nodes, and 39% in spleen were donor-derived (Fig 1). At 60 through 180 days after HCT, the levels of donor Ly-5.1 engraftment in blood, lymph nodes, and spleens of recipients of 10 mg/kg of busulfan were significantly less than values observed in mice transplanted after at least 20 mg/kg (P < .05, two-sample t-test).

In contrast, higher doses of busulfan provided more rapid and complete lymphoid reconstitution by congenic donor cells. In the peripheral blood of Ly-5.2 mice 30 days after HCT following 20 mg/kg of busulfan, greater than 50% of lymphocytes were identified as donor-derived Ly-5.1 cells by flow cytometry. Higher percentages of Ly-5.1 lymphocytes were observed 30 days after HCT in the blood of animals given higher doses of busulfan, ranging from 66% at 35 mg/kg to 85% at 100 mg/kg. The percentage of donor congenic cells in radiation chimeras was 82% at 30 days after HCT (Fig 1A). Sixty days after transplant, 68% of peripheral blood lymphocytes were Ly-5.1 in recipients of 20 mg/kg of the drug, 75% to 80% after 35 mg/kg to 80 mg/kg, and 88% after 100 mg/kg of busulfan. Ninety days after HCT, approximately 90% to 95% of peripheral blood lymphocytes were donor-derived in mice given at least 20 mg/kg of busulfan before transplant, similar to that seen in TBI-conditioned recipients, and remained stable at these levels as late as 180 days after HCT (Fig 1A).

In the lymph nodes, a substantial percentage of donor Ly-5.1 lymphocytes were detected 30 days after HCT: 43% after 20 mg/kg of busulfan, 57% after 35 mg/kg, 65% to 68% after 50 mg/kg to 80 mg/kg, and 81% after 100 mg/kg (Fig 1B). In TBI-conditioned recipients, 77% of lymphocytes from lymph nodes were donor-derived at 30 days after HCT. Sixty days after transplantation, donor cells accounted for 57% of lymphocytes in lymph nodes of mice conditioned with 20 mg/kg of busulfan, 78% after 35 mg/kg, and 84% to 90% after 50 mg/kg to 100 mg/kg; 90% of donor lymphocytes were identified in lymph nodes of radiation chimeras at that time. Three months after HCT, stable congenic lymphoid engraftment was seen in mice given at least 20 mg/kg of busulfan, with 90% to 95% Ly-5.1+ cells in lymph nodes, again similar to that observed after pretransplant conditioning with TBI. Long-term repopulation at these levels of donor lymphocytes was seen as late as 180 days after HCT, with greater percentages of Ly-5.1 cells in animals that received 50 mg/kg or more of busulfan before transplant.

In the spleens of Ly-5.2 mice transplanted 30 days earlier, 54% of lymphoid cells were donor Ly-5.1 in mice given 20 mg/kg of busulfan, 62% to 66% in recipients of 35 mg/kg to 80 mg/kg, and 77% in animals given 100 mg/kg (Fig 1C);
the spleens of TBI-conditioned recipients demonstrated approximately 79% donor lymphocytes. As seen in lymph nodes, there was also a dose-dependent increase in the percentage of Ly-5.1 lymphocytes in spleens of busulfan-conditioned mice; 60 days after HCT, donor cells accounted for 58% of the splenic lymphocytes in mice transplanted after 20 mg/kg of busulfan, 72% after 35 mg/kg, 78% to 85% after 50 mg/kg to 80 mg/kg, and 89% to 90% after either 100 mg/kg of busulfan or TBI. As observed in peripheral blood and lymph nodes, the percentages of donor cells increased to 87% to 90% by 90 days after HCT in animals given at least 20 mg/kg or more of busulfan, compared with 91% in radiation chimeras. Long-term stable repopulation by congenic Ly-5.1 lymphocytes also occurred in spleens of busulfan-conditioned mice; 6 months after transplant, donor splenic lymphocytes accounted for 86% to 87% of the cells in 20 mg/kg or 35 mg/kg recipients, 91% to 93% in mice given 50 mg/kg to 80 mg/kg, and 96% after 100 mg/kg (Fig 1C).

**DISCUSSION**

Busulfan is not classically described as an immunosuppressive agent and cannot be used alone as conditioning for allogeneic BMT. Some morphological studies in rodents given sublethal doses of busulfan without BMT or HCT suggest modest effects of the drug on lymphoid tissues. For example, small lymphocytes in rat marrow decrease to 20%
to 30% of control by 2 days after administration of high-dose busulfan, but quantitative morphological alterations in lymph nodes, spleen, or Peyer’s patches of busulfan-treated animals is minimal. In humans with solid tumors given single high doses of busulfan and autologous marrow infusions, total lymphocyte counts and lymphocyte subpopulations are largely unaffected despite substantial myelosuppression, although assays of immune function were not performed in these patients.

The effects of busulfan on immune function are inconclusive. Preimmunization treatment with busulfan suppresses antibody production in mice, but not in rats. Spleen cells from mice given single high doses of busulfan have impaired plaque-forming responses to sheep erythrocytes after transfer into syngeneic cyclophosphamide-treated recipients, suggesting disproportionately greater effects on B-lineage lymphocytes. As the busulfan doses used in our studies are substantially toxic to hematopoietic cells, these observations could also be consistent with effects of the drug on nonlymphoid tissues such as antigen-presenting cells. In contrast, the lack of any substantial inhibition by busulfan on cell-mediated immunity is demonstrated by rejection of allogeneic skin or bone marrow grafts, preservation of contact hypersensitivity responses, and more rapid recovery of helper T-lymphocyte responses in busulfan-treated rodents.

The use of the Ly-5 congenic murine transplant model in these studies allows the assessment of lymphoid recovery by donor cells without confounding variables such as allogeneic graft-versus-host reactions or administration of immunosuppressive agents to abrogate graft rejection by the host immune system. Using karyotypic analyses, Massa et al have shown the reconstitution by donor pluripotent hematopoietic cells (colony-forming unit-spleen [CFU-S]) in mice given syngeneic BMT after 50 mg/kg of busulfan; similar to our observations with lymphocyte repopulation, more than 90% of spleen colonies were donor-derived by 10 weeks after BMT. However, this study did not specifically examine lymphoid tissues for donor male cells and introduced the additional variable of potential rejection of donor male grafts by female anti-HY immune responses. We have shown that essentially complete reconstitution of lymphoid tissues by transplanted congenic marrow- and spleen-derived stem cells occurs in mice pretreated with at least 20 mg/kg of busulfan, but that engraftment is slower and incomplete at a lower dose (10 mg/kg). The posttransplant reconstitution by donor lymphocytes in mice given the highest doses of busulfan is similar to that observed after TBI, a modality that is both profoundly immunosuppressive and myeloablative. Furthermore, the kinetics and extent of lymphoid reconstitution by congenic Ly-5.1 cells after high-dose busulfan resemble those described for donor-derived hematopoietic repopulation by pluripotent CFU-S in busulfan- or TBI-treated murine recipients of sex-mismatched syngeneic bone marrow grafts.

One promising future application of single-agent high-dose busulfan alone is in the area of gene-insertion or gene-replacement therapy by infusion of autologous marrow cells into which the appropriate cloned functional gene is inserted. This strategy is especially attractive in conditions such as lysosomal storage diseases, for which many of the specific hydrolase genes have already been cloned. Although myeloablation will almost certainly be required for engraftment and proliferation of these modified autologous stem cells, immunosuppression may not be necessary. Since high-dose busulfan is profoundly myeloablative, lacks overt immunosuppressive properties, and is associated with sustained engraftment of and lymphoid reconstitution by donor congenic (syngeneic) cells, it may well be a satisfactory single-agent conditioning regimen for transplantation of genetically modified autologous stem cells. As lymphoid reconstitution after busulfan is prompt and, at least in humans, not associated with substantial effects on lymphocyte subpopulations, the posttransplant immunodeficiency in recipients of high-dose busulfan alone may also be shorter and less profound than that observed after administration of combinations of busulfan and cyclophosphamide or cyclophosphamide and TBI, as currently used in allogeneic and autologous BMT. Recent encouraging reports of successful HCT after high-dose busulfan in a murine model of lysosomal storage disease support the examination of this regimen in clinical studies of gene therapy by autologous BMT.

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Lymphoid reconstitution after transplantation of congenic hematopoietic cells in busulfan-treated mice

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