Graft-Host Tolerance in Bone Marrow Transplant Chimeras. Absence of Graft-Versus-Host Disease Is Associated With Unresponsiveness to Minor Histocompatibility Antigens Expressed by All Tissues

By Sylvie Brochu, Chantal Baron, Robert Bélanger, and Claude Perreault

Because bone marrow (BM) transplantation is used with increasing frequency, it is important to elucidate the mechanisms involved in the establishment of tolerance to host minor histocompatibility antigens (MiHA) in recipients transplanted with T-cell–undepleted marrow grafts. We have previously shown that BM chimeras transplanted across MiHA barriers showed specific unresponsiveness to MiHA expressed on recipient-type concanavalin A blasts. Because expression of many MiHA is tissue-specific, we wanted to determine if chimera T lymphocytes would be tolerant to MiHA expressed by all host tissues and organs. To investigate this issue, we measured in vivo proliferation of lymphoid cells from normal C57BL/10 (B10) mice and (B10 → LP) chimeras in tissues and organs of lethally irradiated syngeneic and allogeneic recipients. Donor B10 cells were either untreated, or depleted with anti-Thy-1.2, anti-CD4, or anti-CD8 antibodies. Transplantation of B10 cells in LP recipients triggered an important T-cell–dependent 121I-dUrd uptake in several organs that involved both CD4+ and CD8+ cells. Using Thy-1–congenic mice we showed that in long-term chimeras practically all CD4+ and CD8+ T lymphocytes were derived from hematopoietic progenitors and not from mature T cells present in the BM graft. When (B10 → LP) BM chimeras were injected to secondary recipients, no proliferation was observed in any organ of LP hosts whereas normal proliferation was seen in H-2b allogeneic hosts. Thus, in these BM chimeras, tolerance encompasses MiHA expressed by all organs.

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ALTHOUGH bone marrow transplantation (BMT) is being used with increasing frequency, particularly for treatment of malignant hematologic disorders, graft-versus-host disease (GVHD) still represents a major barrier and, despite intensive research, little progress has been accomplished during the last few years. Thus, T-cell depletion, the most straightforward approach to prevent GVHD, has proved unsatisfactory because it is associated with increased rates of graft rejection and leukemic relapse. Clinical and experimental observations have shown that graft-host tolerance could sometimes be obtained after BMT across minor histocompatibility antigen (MiHA) barriers but the mechanisms involved were poorly defined and the occurrence of GVHD remained unpredictable, because it was observed in some but not all donor/recipient combinations.

The development of graft-host tolerance in BM chimeras reconstituted with T-lymphocyte–undepleted hematopoietic cells is probably more demanding than the acquisition of self-tolerance because both mature T cells and immature thymocytes encounter host antigen. The most controversial issue relates to the involvement of peripheral (extrathymic) mechanisms in this process. On the one hand studies with transgenic mice suggest that expression of histocompatibility antigen on peripheral epithelial cells can induce tolerance by deletion or inactivation. On the other hand, recent studies by Sprent et al challenged this view. Indeed, tolerance was not apparent when thymectomized parent → F1 chimeras were given parental strain thymus grafts. This last experiment suggests that the expression of host H-2 antigen in the extrathymic environment of chimeras is not tolerogenic for mature T cells.

We have developed a model in which C57BL/6J (B6) or C57BL/10SnJ (B10) BM cells were transplanted in lethally irradiated LP/J (LP) recipients to investigate the mechanisms responsible for tolerance to host MiHA. Although addition of 1 to 5 × 10^6 spleen cells (as a source of T lymphocytes) to the BM inoculum provoked a rapidly lethal GVHD, transplantation with only 10^6 unmanipulated B6 or B10 BM cells (containing 2 to 3 × 10^7 T cells) yielded healthy (B6→LP) or (B10→LP) chimeras. Recipient mice were complete donor-type chimeras with no signs of graft rejection, mixed chimerism or GVHD. They had a normal survival rate and showed excellent immune reconstitution. Detailed studies of (B10 → LP) chimera cytotoxic T-lymphocyte (CTL) responses to single or multiple MiHA showed that they were specifically unresponsive to host(LP)- and donor(B10)-type MiHA but showed normal responses to third-party MiHA. However, these studies were only informative with regard to MiHA expressed on T lymphoblast cells, ie, concanavalin A (Con A) blast targets used for in vitro experiments. Because nonhematopoietic cells are the critical targets of GVH reaction and expression of most MiHA appears to be tissue-specific and not ubiquitous, it was important to determine whether grafted T cells became tolerant to MiHA expressed by all tissues and organs. Therefore, using 121I-dUrd incorporation assay, we evaluated in situ proliferative response of grafted cells from B10 or (B10 → LP) chimera donors in several host tissues and organs after their injection to lethally irradiated B10, LP, or third-party recipients.

Our results showed that disparity for multiple MiHA induced marked in vivo proliferation of transferred T cells that could be detected and measured in most lymphoid and nonlymphoid organs. More importantly, we showed that (B10 → LP) chimera T cells were fully tolerant to host (LP) MiHA expressed on various nonhematopoietic organs.

MATERIALS AND METHODS

Mice and BM chimeras. The following strains of H-2b mice were used throughout these studies: C57BL/6J (Thy-1.2, H-2b), C57BL/10SnJ (B10), and C3H/HeN (C3H).

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C57BL/10Sn (Mls\(\text{s}\)), B6.PL-Thy-1/Cy (B6-Thy-1.1), LP/J (Mls\(\text{s}\)), C3H.SW/S (Mls\(\text{s}\)), 129/J (Mls uncharacterized), and A.BY/Sn (Mls\(\text{s}\)). They are mutually unresponsive in primary mixed lymphocyte reaction because H-2\(^b\) is a nonstimulatory haplotype in the recognition of Mls. Two strains of H-2\(^b\) mice were also used: B10.BR/SgSn (Mls\(\text{s}\)) and C3H/HeJ (Mls\(\text{s}\)). Adult male mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a conventional facility. Irradiated (9.5 Gy) LP or B6 mice injected with \(10^7\) unmanipulated B10 BM cells are referred to as (B10 \rightarrow LP) and (B10 \rightarrow B10) chimeras, respectively. We used these chimeras as source of donor cells 100 to 150 days posttransplant because we had previously shown that chimeras were immuno-competent at that time. All chimeras were between 20 and 30 weeks of age when studied. Other mice used as cell donor and/or irradiated recipients were between 6 and 16 weeks of age.

**Origin of CD\(^4\) and CD\(^8\) T cells in BM chimeras.** Standard BM chimeras, described in the preceding paragraph, were reconstituted with \(10^7\) BM cells containing 2.5 \(\times 10^5\) T cells. To determine if in each long-term BM chimeras, T cells were derived from hematopoietic progenitors or were the progeny of mature T cells present in the graft, we transplanted irradiated LP host mice with a combination of Thy-1-congenic BM stem cells (T-depleted BM cells) and mature T cells (lymph node [LN] cells). Thus, irradiated LP recipients were injected with an inoculum containing \(10^7\) B6 BM stem cells and 3 \(\times 10^7\) B6-Thy-1.1 LN cells (containing 2.5 \(\times 10^5\) T cells) or 10\(^7\) B6-Thy-1.1 BM stem cells and 3 \(\times 10^7\) B6 LN cells.

Auxiliary and cervical LN from B6-Thy-1.1 or B6 mice were collected, teased apart, and washed. BM collected from B6 or B6-Thy-1.1 donors was T cell depleted with specific anti-Thy-1.2 or Thy-1.1 monoclonal antibodies (MoAbs); respectively. BM and LN cells were mixed and injected intravenously.

**Radioactive, chemical products and monoclonal antibodies (MoAbs).** 5\(\text{-}^{[3]}\)Hiodo-2-deoxyuridine (\(\text{\textsuperscript{3}}\text{H}\text{-dUrd}\); specific activity 6.25 Ci/mg) was obtained from NEN, Du Pont (Markham, Ontario, Canada); fluoro-2-dUrd was purchased from Sigma (St. Louis, MO). Cytotoxic MoAbs anti-L3/T4 (YTS 191.1, rat IgG2b), anti-Ly2.2 [AD4(15); mouse IgM], anti-Thy-1.2 (5a-8; mouse IgG2b) and anti-Thy-1.1 (T11D7e; mouse IgM), and specific phycoerythrin (PE)-conjugated MoAbs anti-L3/T4 (YTS 191.1, rat IgG2b), anti-Ly2.2 (YTS 169.4; rat IgG2b), and their isotopic PE controls were obtained from Cedarlane (Hornby, Ontario, Canada); specific fluororescin isothiocyanate (FITC)-conjugated MoAbs anti-L3/T4 (KT9; rat IgG2c), anti-Ly2.2 (KT15; rat IgG2a), anti-Thy-1.1 (MRC OX-7; rat IgG1), and their isotopic FITC controls were purchased from Serotec (Toronto, Ontario, Canada) and Pharmingen (San Diego, CA); specific FITC-conjugated MoAbs anti-Thy-1.2 (T5; mouse IgM) were purchased from ICN.

**Cell transplantation and GVHD induction.** Mice were transplanted as described previously. Briefly, recipient mice received 9.5 Gy total body irradiation from a source at a dose rate of 128 cGy/min 6 to 18 hours before their reconstitution with an inoculum of transfused BM cells. Under these conditions all types of H-2\(^d\) recipients grafted with untreated B10 cells die of severe GVHD.\(^{21}\)

**Measurement of grafted cell proliferative activity.** We measured in vivo proliferation of grafted cells (Fig 1) in several host tissues and organs using a method originally developed by Spach and Motta.\(^{22}\) Briefly, on day 6 (H-2\(^d\) mice) or on day 8 (H-2\(^d\) mice) after irradiation and cell transplantation, mice received an intraperitoneal (ip) injection of fluoro-2-dUrd 125i.dUrd (6.25 CU/mg) was obtained from NEN, Du Pont (Markham, Ontario, Canada); fluoro-2-dUrd was purchased from Sigma (St. Louis, MO). Cytotoxic MoAbs anti-L3/T4 (YTS 191.1, rat IgG2b), anti-Ly2.2 [AD4(15); mouse IgM], anti-Thy-1.2 (5a-8; mouse IgG2b) and anti-Thy-1.1 monoclonal antibodies (MoAbs); respectively. BM and LN cells were mixed and injected intravenously.

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**Depletion of Thy-1\(^+\), CD\(^4\), and CD\(^8\) cells.** Cells to be treated were resuspended at a concentration of \(1 \times 10^7\) cells/mL and incubated with depleting MoAb at 4°C for 1 hour. They were then pelleted by centrifugation, resuspended in rabbit serum as a source of complement, and incubated at 37°C for 1 hour. Cell suspensions were washed three times, analyzed for efficacy of depletion by direct cytometry, and then adjusted for injection. For proliferative activity assays, spleen and BM B10 cells were depleted separately and mixed in adequate concentration just before injection. To keep constant the number of non-T cells in each inoculum, the total quantity of sponge cells (2.5 \(\times 10^7\) in recipients of unmanipulated graft) injected to recipients of Thy-1.2, CD-4 and CD-8--depleted grafts was adjusted to 1.7 \(\times 10^7\), 2.07 \(\times 10^7\), and 2.2 \(\times 10^7\), respectively.

**Immunofluorescence staining and fluorescence-activated cell sorter (FACS) analysis.** Single- and double-immunofluorescence staining were performed directly with FITC and PE-conjugated MoAbs. Five hundred thousand cells per sample were incubated for 25 minutes at 4°C with the appropriate FITC- and/or PE-conjugated MoAbs (double labeling analysis) diluted in a final volume of 125 mL PBS. After 3 washes in PBS, propidium-iodine negative cells were analyzed for surface fluorescence on a FACStar plus flow cytometer (Becton Dickinson) and the data were processed using the lysys II program (Becton Dickinson). Nonspecific binding of
Fig 2. \(^{131}I\)-dUrd incorporation in 21 organs after irradiation and transplantation of hematopoietic cells (\(10^7\) BM and \(2.5 \times 10^7\) spleen cells) from B10 donors to lethally irradiated LP (vertical bars) recipients. LP mice were either transplanted with unmanipulated (\(\square\)) or with Thy-1-depleted hematopoietic cells (\(\square\)) or left unreconstituted (\(\square\)). On day 8, recipients were injected with fluoro-2-dUrd and 1 hour later were labeled by a single ip injection of \(^{131}I\)-dUrd. One hour after labeling, mice were killed and incorporated radioactivity was measured. Each bar represents the mean \(\pm\) 1 SD cpm for three to five experiments except for Thy-1 depletion, which was done only once. Clear area shows the mean \(\pm\) 1 SD cpm observed in syngeneic B10 recipients of unmanipulated graft.
RESULTS

Proliferative activity of grafted cells from B10 donors. 

$^{125}$I-dUrd incorporation in syngeneic B10 recipients showed major differences among the 21 organs tested as it ranged from $10^3$ to $10^5$ cpm/organ. Similar results were obtained in six experiments (Fig 2). When $^{125}$I-dUrd uptake was measured in allogeneic LP recipients and compared with syngeneic recipients, three patterns were observed. In most organs (thereafter referred to as type 1), $^{125}$I-dUrd uptake was greater in allogeneic than in syngeneic recipients: testes, heart, lungs, kidneys, pancreas, liver, muscles, skin, esophagus, and LN. In type 2 organs (BM, spleen, and gastrointestinal tract [GIT]) no allogeneic/syngeneic difference was detected. The thymus was the sole organ where incorporation was lower in allogeneic than in syngeneic recipients (type 3).

T-cell depletion of B10 inoculum before injection to irradiated LP recipients decreased $^{125}$I-dUrd uptake in type 1 organs down to levels observed in syngeneic hosts, but did not influence incorporation in type 2 organs. T-cell–indepen-
Fig 5. 3H-dUrd incorporation in lymphoid and nonlymphoid tissues from four strains of lethally irradiated mice grafted with hematopoietic cells from (B10 → LP) chimeras. Mice were treated as in Fig 2 and results were expressed as allogeneic/syngeneic ratios.

Fig 6. 3H-dUrd incorporation in lymphoid and nonlymphoid tissues from two types of lethally irradiated MHC-incompatible recipients grafted with hematopoietic cells from (A) B10 donors and (B) syngeneic (B10 → B10) and (C) allogeneic (B10 → LP) chimeras. Two to three mice per group were treated as in Fig 2 on day 6 and results were expressed as allogeneic/syngeneic ratios.
dent uptake in type 2 organs could be ascribed to two mechanisms. 125I-dUrd incorporation in hematopoietic organs (BM, spleen) was abrogated in irradiated ungrafted LP recipients and therefore resulted from the proliferation of donor-derived hematopoietic cells. On the other hand, high uptake in the GIT was independent of donor cells since it was observed in irradiated unconstituted hosts. High GIT uptake may be caused by repair from the radiation induced damage or to incorporation by intestinal and gastric glandular secretions.

When B10 hematopoietic cells were injected to three other types of MiHA-incompatible H-2k recipients (C3H.SW, 129, and A.BY), organ-specific 125I-dUrd uptake showed some strain to strain variations but the same three patterns were observed (Fig 3). Thus, recognition of allogeneic MiHA induced a significant T cell, or at least T-dependent, proliferation in LN and a large variety of nonhematopoietic organs in the four types of H-2k recipients. High T-cell–independent 125I-dUrd uptake precluded evaluation of T-cell responses in type 2 organs.

Role of T-cell subsets. The effect of in vitro depletion of CD4+ or CD8+ lymphocytes on 125I-dUrd uptake by type 1 organs was evaluated in irradiated LP recipients grafted with B10 hematopoietic cells (Fig 4). Depletion of both CD4+ and CD8+ lymphocytes decreased, to a variable extent, proliferative activity in 9 to 10 of 12 organs tested. CD4-depletion had a greater impact on some organs but the reverse was true for others. Similar results were obtained in 129 recipients but a different organ pattern was observed (data not shown).

Proliferation of transplanted (B10 → LP) chimera cells. After transplantation of (B10 → LP) chimera cells into irradiated B10 recipients, the tissue-specific pattern of 125I-dUrd incorporation was similar to the one obtained with normal B10 donors (data not shown). However, results were strikingly different when chimera cells were transplanted in secondary LP recipients (Fig 5). Contrary to what was observed in recipients of B10 cells, no T-cell–dependent proliferation was seen in any type 1 organ of LP mice transplanted with chimera cells (ie, A/S ratio < 3). The pattern of 125I-dUrd incorporation was similar to the one observed after syngeneic transplantation or T-depleted allogeneic transplantation. Thus, in vivo, (B10 → LP) chimera cells were totally unresponsive to MiHA expressed on all tissues from LP mice.

When (B10 → LP) chimera hematopoietic cells were transplanted in third-party recipients such as C3H.SW, 129, and A.BY, T-cell–specific proliferation in type 1 organs was much lower than what was measured in recipients of B10 cells (compare Figs 3 and 5). T-cell–dependent uptake was completely abrogated in some organs and significantly decreased in others. Although we had shown with a number of in vivo and in vitro tests that our 100-day-old BM chimeras were immunocompetent,3 in healthy BM patients some T-cell responses take up to four years to normalize.5 To determine if hematopoietic (B10 → LP) chimera cells have the capacity to generate a normal proliferative response in vivo, they were transplanted into irradiated major histocompatibility complex (MHC)-incompatible recipients with or without minor lymphocyte stimulating loci (Mls) difference (Fig 6). The proliferation of (B10 → LP) chimera cells in type 1 organs was normal when compared with that of (B10 → B10) chimera and B10 donor cells. The pattern of 125I-dUrd incorporation showed the same level of uptake in all organs. Thus, although responsiveness of chimera cells to third-party MiHA was significantly depressed, their responsiveness to MHC antigens was normal.

Origin of T cells in (B10 → LP) BM chimeras. After observation that cells from long-term BM chimeras were tolerant to all MiHA expressed by host cells, we questioned the origin of their T lymphocytes. Do they derive from maturation of hematopoietic progenitors or from expansion of mature T cells present in the original BM inoculum? Table 1 shows the results of two sets of experiments using a combination of Thy-1–congenic hematopoietic progenitors (T-depleted BM cells) and mature T cells (LN cells). Phenotyping with anti–Thy-1.1 and anti–Thy-1.2 MoAbs showed that all thymic cells were derived from hematopoietic progenitors. In the spleen, 92% to 96% of the CD4+ and 78% to 88% of the CD8+ T lymphocytes were also derived from hematopoietic progenitors whereas a minority had the same phenotype as LN cells present in grafted cells.

DISCUSSION

The first evidence that “in vivo mixed lymphocyte reaction” could detect MiHA incompatibilities was provided some years ago by Spach and Motd’in a strongly Mls stimulatory H-2k strain combination: (B10.D2 × DBA/2)F1 (Mls8 x Mls8) recipients were reconstituted with B10.D2 (Mls8) hematopoietic cells. At this time, Mls superantigens were confused with MiHA under the imprecise denomination of “non-MHC antigen.” The present studies confirm and expand these original observations by showing that disparity for multiple MiHA can induce significant and measurable in situ proliferation of transplanted T cells in Mls nonstimulatory donor/recipient combinations. We detected T-cell proliferation in all extrathymic lymphoid and nonlymphoid organs, except for the GIT and hematopoietic organs (BM, spleen) where the high basal 125I-dUrd uptake precluded further evaluation. This is consistent with histologic description of tissues obtained 7 to 8 days after BMT across various types of MiHA barriers showing widespread lymphocytic infiltrates in the skin, liver, pancreas, lungs, and kidneys.15,26,27 The thymus was an exception because we observed a decreased T-cell–dependent 125I-dUrd uptake in allogeneic recipients compared with syngeneic recipients. We hypothesized that maturation in an MiHA-incompatible thymus may possibly increase negative selection by causing deletion of T-cell clones recognizing both donor- and host-type MiHA expressed on hematopoietic antigen-presenting cells and epithelial cells, respectively. However, this hypothesis remains highly speculative.

Our depletion experiments with anti–CD4 and anti–CD8 MoAbs provided evidence that both T-cell subsets responded to allogeneic MiHA after BMT. The relative contribution of each lymphocyte subset showed significant variation among various organs. This is consistent with a number of observations: firstly, MiHA peptides are associated with both MHC class I and class II molecules,17,28 secondly, both CD4+ and CD8+ T cells can contribute to anti–MiHA GVHD29,30, and thirdly, both subsets have significant proliferative potential.31

The aim of the present work was to characterize the state
of tolerance to host MiHA in lethally irradiated BM chimeras reconstituted with T-cell–undepleted graft. Specifically, we wanted to determine if (B10 → LP) chimeras’ T cells would be unresponsive to MiHA expressed on all tissues of the LP host. Our analysis of \( ^{3}H \)-dUrd incorporation in tissues and organs of secondary LP recipients injected with chimeras’ T cells clearly showed that the latter did not proliferate in any organ. This is consistent with previous observations that chimeric cells cannot trigger GVH reaction when injected into secondary LP recipients and are unresponsive to host MiHA when tested in CTL assays against LP Con A blast targets. Thus, according to both in vitro CTL assays and in vivo proliferation studies (Fig 5), BM chimeras are tolerant to MiHA expressed by all organs of the host.

Although the number of MiHA gene differences between various inbred strains of mice is probably greater than 40\(^2\) and the product of many of these can probably stimulate in vivo T-cell proliferation,\(^3\) we must remember that T-cell response to MiHA is characterized by the phenomenon of immunodominance.\(^4,5\)

Table 1. Origin of Thymic and Splenic (B6 → LP) Chimera T Cells

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Irradiated (960 Gy) LP recipient mice were reconstituted with a combination of \( 10^5 \) T-depleted BM stem cells and \( 3 \times 10^5 \) LN cells containing 2.5 \( \times 10^5 \) mature T cells (the same number as found in \( 10^5 \) undepleted BM cells). On day 65 ± 15 after transplantation, lymphocytes isolated from host thymus and spleen were analyzed for Thy-1.1 or Thy-1.2 antigen expression by direct flow cytometry. Results are expressed as the percentage of double-stained lymphocytes for both CD4 and CD8 and Thy-1.1 or Thy-1.2 antigens as determined by double-immunofluorescence staining and corrected for nonspecific binding by isotype-matched control MoAbs.

in vitro unresponsiveness is frequently called “split tolerance.” It is thought to reflect the fact that tolerance induction is a multi-step process that may occur in a step-wise fashion.\(^6,7\) After their first encounter with antigen, T cells may be functionally inactivated but not physically deleted. Anergized T-cells, may persist indefinitely or may be eliminated after subsequent encounters with antigen. The conditions used for CTL assays, ie, in vivo priming followed by in vitro restimulation, may show the presence of anergized/undeleted T cells.

Knowing that (B10 → LP) T cells were tolerant to host MiHA, we wondered if they were derived from mature T cells present in the BM graft, implying that the tolerance state depended on peripheral mechanisms. On the contrary, we found that around day 65 postreconstitution most lymphocytes (about 94% of the CD4\(^+\) and 83% of the CD8\(^+\) T cells) originated from BM progenitors and thus, had an opportunity to get thymic education in the LP host. Our previous work suggested that peripheral tolerizing cells were involved in maintenance of tolerance in BM chimeras.\(^4\) The thymus may also play a crucial role even if hematopoietic cells are donor-derived. Indeed, thymic epithelial cells may cause anergy or deletion of developing thymocytes.\(^8,9\) Moreover, as donor and host are MHC-identical, thymic hematopoietic cells of donor origin might present MiHA synthesized by peripheral cells.\(^10\) Further experiments, requiring production of large numbers of BM chimeras, are underway to compare the functional status of T cells derived from BM progenitors and the progeny of BM graft mature T cells.

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Graft-host tolerance in bone marrow transplant chimeras. Absence of graft-versus-host disease is associated with unresponsiveness to minor histocompatibility antigens expressed by all tissues

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