DEFECTIVE LYMPHOPOIESIS IN THE BONE MARROW OF MOTHEATEN (*me/me*) AND VIABLE MOTHEATEN (*me^v/me^v*) MUTANT MICE

III. Normal Mouse Bone Marrow Cells Enable *me^v/me^v* Prothymocytes to Generate Thymocytes after Intravenous Transfer

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Mice homozygous for the recessive single gene mutations motheaten (*me/me*) and viable motheaten (*me^v/me^v*) develop autoimmune and severe combined immunodeficiency disorders (1). Previous results from our laboratory (2) demonstrated the absence of detectable prothymocyte activity in the bone marrow of *me/me* and *me^v/me^v* mice after intravenous injection into sublethally irradiated recipients. However, when the intrathymic (i.t.) adoptive transfer assay for prothymocytes (3) was used, *me/me* and *me^v/me^v* thymocytes were generated in numbers equivalent to those produced by bone marrow cells from apparently normal +/− littermates (2). These results suggested that *me/me* and *me^v/me^v* prothymocytes are present in normal numbers, but that they are unable to home effectively to the thymus via the blood.

In another study (4), we demonstrated that *me^v/me^v* bone marrow can generate presumptive T-lineage terminal deoxynucleotidyl transferase–positive (TdT^+) cells in vitro when cultured on bone marrow stromal cell feeder layers from normal mice; but that feeder layers from *me/me* and *me^v/me^v* mice fail to support the generation of TdT^+ bone marrow cells from normal animals. These results suggested that at least some of the defective lymphopoiesis seen in these mutant mice may be secondary to abnormalities in the bone marrow microenvironment (5).

In the present study, the results of mixing experiments suggest that a population of radiosensitive bone marrow cells from normal mice can restore the ability of *me^v/me^v* prothymocytes to generate thymocytes after intravenous injection. The results provide further evidence that a microenvironmental defect may be responsible for the developmental abnormalities of lymphoid precursor cells in *me^v/me^v* mice, and suggest that the *me^v/me^v* mutant mouse may be a useful model

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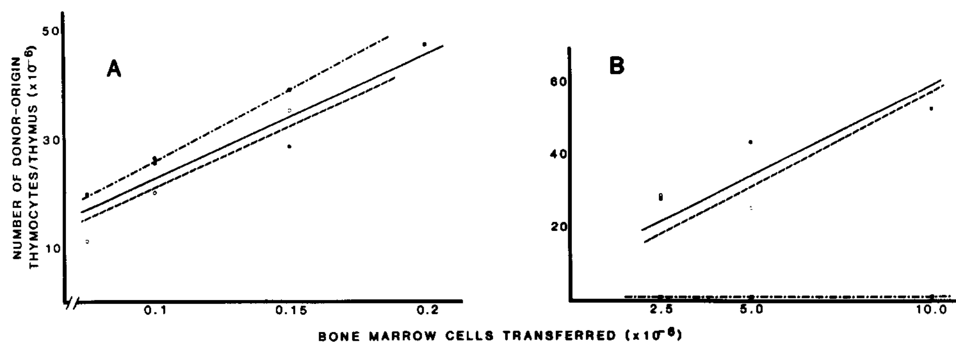

FIGURE 1. Generation of donor-origin (Ly-1.2) thymocytes as a function of the dose of C57BL/6 +/+ wild-type (●), C57BL/6J-*me*^{v}/*me*^{v} (■) or C57BL/6J +/− littermate (○) bone marrow cells injected (*A*) intrathymically or (*B*) intravenously into irradiated (600 rad) C57BL/6-Ly-1.1 wild-type recipients 17 d previously. Each point represents the mean of three animals. The best fit lines were determined by linear regression analysis: (*A*) *me*^{v}/*me*^{v}, r^2 = 1.00, slope = 257.9; +/− littermate, r^2 = 0.96, slope = 230.8; +/+ wild-type, r^2 = 0.96, slope = 222.6; (*B*) +/− littermate, r^2 = 0.90, slope = 5.28; +/+ wild-type, r^2 = 0.84, slope = 4.99.

with which to identify the cells and/or factors in bone marrow that help to regulate the differentiation, proliferation, and/or migration of prothymocytes.

Materials and Methods

Animals. 5–7-wk-old C57BL/6J-*me*^{v}/*me*^{v} mice (H-2^b, Ly 1.2) and their phenotypically normal littermate controls (approximately ⅔ +/*me*^{v} and ⅓ +/+) were obtained from the colony maintained by L. Shultz at The Jackson Laboratory, Bar Harbor, ME. Only *me*^{v}/*me*^{v} mice have been used in the present study, since they have longer lifespans than do the *me*/*me* mice (1). C57BL/6 wild-type (+/+) mice (H-2^b, Ly-1.2) were obtained from the Animal Genetics and Production Branch, National Cancer Institute, Frederick, MD. Breeding pairs of congenic C57BL/6-Ly-1.1 mice (wild-type, +/+) (H-2^b, Ly-1.1) were obtained from Dr. Edward Boyse, Sloan-Kettering, New York.

Immunofluorescence. Thymocyte cell suspensions were labeled with mAbs to the mouse Ly-1.1 or Ly-1.2 alloantigens (New England Nuclear, Boston, MA), to distinguish between donor- and host-origin cells, and developed with a fluorescein-conjugated F(ab′)$_2$ fragment of a goat anti–mouse IgG (heavy and light chain specific) (Cappel Laboratories, Cochranville, PA) (2). Labeled cells were analyzed on a FACS IV (Becton Dickinson & Co., Sunnyvale, CA).

Adoptive Transfer Assays for Prothymocytes. The intravenous and intrathymic quantitative adoptive transfer assays for prothymocytes have been described previously (3). In brief, irradiated recipients (600 rad) were injected with graded doses of bone marrow cells intravenously into the tail vein or intrathymically into each thymic lobe. At day 17 after cell transfer, the recipients were sacrificed and the numbers of donor- and host-origin thymocytes were determined by immunofluorescence on the FACS.

Results

Prothymocyte Activity of Bone Marrow Cells from me^v*/me*^{v}*, +/− Littermate, and +/+* Wild-type Mice. To confirm our previous observations (2) and to ensure that +/− littermate mice can serve as normal controls despite the possible presence of a recessive *me*^{v} allele, we have compared the relative prothymocyte activities of *me*^{v}/*me*^{v}, +/− littermate, and +/+ wild-type C57BL/6-Ly-1.2 bone marrow cells after injection intrathymically and intravenously into irradiated (600 rad) C57BL/6-Ly-1.1 mice. Results of the intrathymic assay (Fig. 1*A*) show that the

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TABLE I

Inability of me^v/me^v Prothymocytes Recovered from the Bone Marrow of An Intermediate +/+ Wild-type Recipient to Generate Thymocytes when Transferred Intravenously into a Second Wild-type Recipient

Origin of transferred cells	Primary recipient of bone marrow cell transfer		Secondary recipient of bone marrow cell transfer		Number of thymocytes per thymus in secondary recipients ($\times 10^{-6}$)	
	Route	Number of cells	Route	Number of cells	Donor-origin	Host-origin
+/− Littermate	i.v.	10×10^6	i.v.	14.0×10^6	58.4 ± 14.1	2.6 ± 4.1
			i.t.	2.5×10^6	74.5 ± 25.5	2.2
me^v/me^v	i.v.	10×10^6	i.v.	14.0×10^6	<1	2.4 ± 3.3
			i.t.	2.5×10^6	76.9 ± 5.4	1.9 ± 0.6

Irradiated (600 rad) C57BL/6-Ly 1.1 wild-type (+/+) mice were injected i.v. with me^v/me^v (Ly-1.2) or +/− littermate (Ly-1.2) bone marrow cells. 17 d later, bone marrow cells from these primary recipients were transferred i.v. or i.t. into irradiated (600 rad) C57BL/6-Ly-1.1 and C57BL/6-Ly-1.2 wild-type secondary recipients. Donor-origin (Ly-1.2) and host-origin (Ly-1.1) thymocytes were quantified by FACS analysis 17 d later. Results are the means ± SD of three animals per group.

number of donor-origin thymocytes generated at day 17 by all three sources of bone marrow cells was directly proportional to the number of cells transferred over the dose range of 0.075×10^6 to 0.2×10^6 cells. Moreover, no significant differences were observed between the numbers of donor-origin thymocytes generated by each group at any given cell dose.

Like the intrathymic assay, results of the intravenous assay (Fig. 1 *B*) show that the number of donor-origin thymocytes generated at day 17 were directly proportional to the number of +/− littermate or +/+ wild-type bone marrow cells transferred over the dose range of 2.5×10^6 to 10×10^6 cells; and that there were no significant differences in the numbers of donor-origin thymocytes generated by either group at any given cell dose. However, unlike the intrathymic assay, as many as 10×10^6 me^v/me^v bone marrow cells failed to generate any detectable donor-origin thymocytes in the intravenous assay system.

Passage of me^v/me^v Bone Marrow Cells Through Irradiated +/+ Wild-type Recipients Does Not Restore Prothymocyte Activity in the Intravenous Assay. 10^6 me^v/me^v (Ly-1.2) or +/− littermate (Ly-1.2) bone marrow cells were transferred intravenously into sublethally irradiated (600 rad) C57BL/6-Ly-1.1 wild-type recipients. 17 d later the bone marrow of these recipient mice was harvested and tested for prothymocyte activity by intravenous and intrathymic injection into sublethally irradiated (600 rad) C57BL/6-Ly-1.1 and C57BL/6-Ly-1.2 secondary recipients. No me^v/me^v-origin (Ly-1.2) thymocytes were detected in the intravenous-injected secondary recipients at day 17; whereas 76.9×10^6 me^v/me^v-origin thymocytes were present in the intrathymic-injected secondary recipients (Table I). In contrast, 58.4×10^6 and 74.5×10^6 donor-origin (Ly-1.2) thymocytes were detected, respectively, in both intravenous-injected and intrathymic-injected secondary recipients when +/− littermate bone marrow cells were passaged. Fewer than 3×10^6 host-origin (Ly-1.1) thymocytes were present after either intravenous or intrathymic injection of bone marrow cells from the primary hosts into Ly-1.2 secondary recipients.

Mixing of me^v/me^v and +/+ Wild-type Bone Marrow Cells Enables me^v/me^v prothy-

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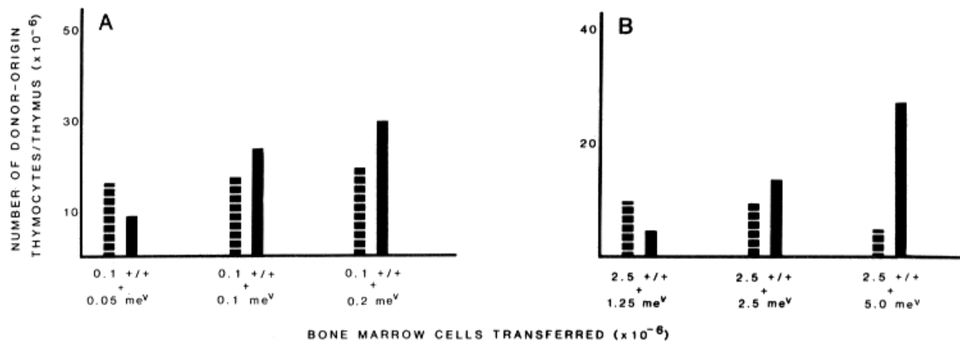

FIGURE 2. Generation of me^{v}/me^{v}-origin (Ly 1.2) (■) and wild-type-origin (Ly-1.1) (▤) thymocytes following the transfer of mixtures of me^{v}/me^{v} and C57BL/6-Ly 1.1 wild-type bone marrow cells (*A*) intrathymically or (*B*) intravenously into irradiated (600 rad) C57BL/6-Ly-1.1 or C57BL/6-Ly-1.2 wild-type recipients, respectively, 17 d previously. The C57BL/6-Ly-1.1 wild-type bone marrow cells were mixed at a constant dose (0.1×10^6 intrathymic or 2.5×10^6 intravenous) with varying doses of me^{v}/me^{v} (Ly-1.2) bone marrow cells. Each value represents the mean of three animals.

mocytes to Generate Thymocytes When Transferred Intravenously. Varying numbers of me^{v}/me^{v} (Ly-1.2) bone marrow cells were mixed with 0.1×10^6 or 2.5×10^6 wild-type (C57BL/6-Ly-1.1) bone marrow cells and injected intrathymically or intravenously, respectively, into irradiated (600 rad) C57BL/6-Ly-1.1 and C57BL/6-Ly-1.2 recipients. As shown in Fig. 2, *A* and *B*, me^{v}/me^{v} thymocytes were generated in a dose-related manner in B6-Ly-1.1 recipients both after intrathymic and intravenous injection. In addition, the number of wild-type origin thymocytes that were generated in B6-Ly-1.2 recipients by the mixture of bone marrow cells was equivalent to that expected if only wild-type bone marrow cells had been injected intrathymically or intravenously (compare Fig. 1, *A* and *B*, with Fig. 2, *A* and *B*).

Discussion

The results of the present study demonstrate that the inability of me^{v}/me^{v} prothymocytes to repopulate the thymus of irradiated recipients after intravenous injection can be corrected by the addition of wild-type bone marrow cells before injection. The simplest explanation for this observation is that a population of normal bone marrow cells (and/or their products), which enable me^{v}/me^{v} prothymocytes to home to and/or differentiate within the thymus, are deficient or defective in me^{v}/me^{v} mice. Although our previous in vitro experiments favor a role for bone marrow macrophages in this process (4), it is also possible that other cell types, including normal prothymocytes (or autocrine factors therefrom), may be involved (but see below). However, it is unlikely that the inability of me^{v}/me^{v} prothymocytes to home effectively to the thymus results from inhibitory cells or factors, since the mixing of me^{v}/me^{v} and wild-type bone marrow cells before intravenous transfer does not decrease the generation of wild-type thymocytes (Fig. 2). Moreover, it is unlikely that a defect within the thymus itself is involved, inasmuch as normal bone marrow cells, injected intravenously, readily repopulate the thymus of irradiated *me/me* and me^{v}/me^{v} mice (1).

Unfortunately, very few host-origin prothymocytes were detected, by either

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the intravenous or the intrathymic adoptive transfer assays, in wild-type recipients of *me*^{v}/*me*^{v} bone marrow cells 17 d after irradiation (Table I). Thus we were not able to determine whether wild-type prothymocytes can correct the developmental defect of *me*^{v}/*me*^{v}-origin prothymocytes. However, it has been observed in other studies (Shultz, L., unpublished observations) that bone marrow cells from *scid* mice, which themselves lack detectable prothymocytes in the intravenous transfer system (1), enable *me*^{v}/*me*^{v} prothymocytes to generate thymocytes in the intravenous transfer system. This suggests that normal prothymocytes are not implicated in this phenomenon.

Whatever its identity, the normal bone marrow cell(s)/factor(s) that restores *me*^{v}/*me*^{v} prothymocyte activity after intravenous transfer appears to be relatively radiosensitive. Not only did *me*^{v}/*me*^{v} bone marrow cells fail to generate thymocytes after intravenous injection into 600 rad irradiated wild-type recipients, but bone marrow cells obtained from these recipients 17 d later also failed to generate *me*^{v}/*me*^{v}-origin thymocytes upon secondary transfer intravenously. Yet these same 17-d postreconstitution bone marrow cells readily generated *me*^{v}/*me*^{v}-origin thymocytes in the intrathymic assay system, thereby indicating that many *me*^{v}/*me*^{v} prothymocytes were present. In more recent experiments we have demonstrated that bone marrow obtained from wild-type mice 72 h after 600 rad whole-body irradiation fails to restore *me*^{v}/*me*^{v} prothymocyte activity in the intravenous transfer system (our unpublished observations). Thus, the putative accessory function rapidly disappears from normal bone marrow after 600 rad and is not replaced within 17 d.

The results of the intrathymic transfer experiments suggest that, once in the thymic microenvironment, *me*^{v}/*me*^{v} prothymocytes no longer require other radiosensitive bone marrow cells to proliferate and/or differentiate. If this is correct, then the failure of *me*^{v}/*me*^{v} prothymocytes to generate thymocytes after intravenous injection is most likely due to their inability to home to the thymus. Alternatively, radiosensitive bone marrow cells may indeed be required to support thymocytopoiesis after prothymocytes enter the thymus, but in *me*^{v}/*me*^{v} mice these cells may themselves be defective in their thymus-homing abilities. Under these circumstances, *me*^{v}/*me*^{v} prothymocytes could have normal thymus-homing capacities and still not be detectable in the intravenous transfer system.

Experiments using combined intrathymic and intravenous transfer of wild-type and *me*^{v}/*me*^{v} bone marrow cells, respectively, have been initiated to distinguish between these alternatives. However, the existence of a thymus-homing defect of prothymocytes and/or other bone marrow cells in *me*^{v}/*me*^{v} mice is further supported by the results of in vitro migration assays, in which only 0.7% of *me*^{v}/*me*^{v} bone marrow cells migrated in response to supernatants of normal thymus, as compared with 8.8% of +/− littermate bone marrow cells (6). In addition, the possible role of Ia$^+$ nonlymphoid bone marrow cells in prothymocyte maturation and migration is being explored. Such Ia$^+$ cells have been shown to help regulate thymocytopoiesis in vivo (7) and in vitro (8), to be relatively radiosensitive (9), and to migrate from bone marrow to thymus (9).

Summary

Bone marrow prothymocytes from *me*/*me* and *me*^{v}/*me*^{v} mutant mice fail to generate thymocytes in irradiated (600 rad) +/+ wild-type recipients after

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intravenous injection. However, these same prothymocytes readily generate thymocytes after intrathymic injection. The results of the present study demonstrate that this apparent defect in the thymus-homing capacity of me^v/me^v prothymocytes can be corrected by mixing irradiated wild-type bone marrow cells with me^v/me^v bone marrow cells before intravenous injection. However, this defect is not corrected by passage of me^v/me^v bone marrow cells through the bone marrow of irradiated wild-type recipients. One interpretation of these results is that the maturation of prothymocytes is reversibly arrested in me^v/me^v mice by a defect in the radiosensitive compartment of the bone marrow microenvironment.

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