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

I. Analysis of Development of Prothymocytes, Early B Lineage Cells, and Terminal Deoxynucleotidyl Transferase–positive Cells

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Mice homozygous for the autosomal recessive single gene allelic mutations, motheaten (*me/me*) and viable motheaten (me^v/me^v), are unique among mutant mice in that they develop autoimmune syndromes in association with severe combined immunodeficiency disorders (SCID) (see 1 for review). Homozygous *me/me* and me^v/me^v mice have mean lifespans of 22 and 61 d, respectively, and can be recognized as early as 1–3 d by the development of severe dermatitis and patchy alopecia (2, 3). Autoimmune abnormalities include hypergammaglobulinemia, the production of multiple autoantibodies, and the development of immune complex glomerulonephritis (3–7). In addition, the dermatitis that characterizes these mutants and the hemorrhagic macrophagic pneumonitis that is the usual cause of death, are suspected to have an autoimmune basis (1).

Despite the evidence (3–8) for polyclonal B cell activation in *me/me* and me^v/me^v mice, these animals are severely immunodeficient. Thus, the number of peripheral B lymphocytes is decreased by approximately two-thirds in these mutants, and although normal numbers of peripheral T cells are present, thymic involution commences by 4 wk of age, antigen-specific cytotoxic T cell activity and mitogen-induced blastogenesis is markedly reduced, and antibody responses to thymic-dependent (and thymic-independent) antigens are undetectable (1–8).

This work is supported by grant AI-09649 from the National Institute of Allergy and Infectious Diseases, by grant CA-20408 from the National Cancer Institute, and by a grant from the University of Connecticut Research Foundation. Preliminary reports have been presented (Greiner, D. L., K. Komschlies, I. Goldschneider, and L. Shultz. 1986. Migratory defect in prothymocytes of motheaten mice. *Proc. VI Internat. Congr. Immunol.* In press; and Goldschneider, I., D. L. Greiner, E. S. Medlock, and L. Shultz. 1986. B cell lineage development in motheaten strain mice: Description of a microenvironmental defect. *Proc. VI Internat. Congr. Immunol.* In press).

The above autoimmune and immunodeficiency disorders in *me/me* and *me^v/me^v* mice have been postulated to result, at least in part, from immunoregulatory imbalances related to the excessive production of a B cell maturation factor (5, 9, 10) and from the hyperactivity of a population of suppressor macrophages (6).

Motheaten (*me/me*) mice have been reported (11) to have markedly reduced numbers of terminal deoxynucleotidyl transferase (TdT)[1]–positive cells in their bone marrow and thymus. This observation is of particular interest in that TdT appears to be selectively expressed by primitive members of both the T and B cell lineages (12–16), and TdT itself has been postulated to be involved in the diversification of antigen receptors and/or in the programmed death of immature, autoreactive lymphocytes (17–19). Moreover, we have observed that abnormalities in the development of TdT$^+$ bone marrow cells and/or thymocytes precede the onset of autoimmunity in several other mutant mouse models, including the NZB, NZB/W F_1 and BSXB models of systemic lupus erythematosis, and the SJL/J and PL/J models of experimental allergic encephalomyelitis (20 and our unpublished observations). Also, it has been noted (1, 21–24) that the predisposition to autoimmunity can be passed from these mutant mice, and from *me/me* and *me^v/me^v* mice, to normal naive recipients by the adoptive transfer of bone marrow cells. Hence, it is possible that the reported abnormalities in the development and function of peripheral T and B cells in *me/me* and *me^v/me^v* mice may similarly be related to the defective development of TdT$^+$ cells and/or other lymphoid precursor cells. This study is designed to provide such information.

Preliminary experiments in our laboratories, using the traditional i.v. adoptive transfer assay system to identify prothymocytes (25), failed to identify cells in the bone marrow of *me/me* or *me^v/me^v* mice that were able to repopulate the thymus of normal irradiated recipients. Therefore, we have used a novel intrathymic (i.t.) adoptive transfer system (26), which is independent of cell migration, to attempt to detect prothymocytes in the bone marrow of these mutant mice. The results indicate that prothymocytes are indeed present in *me/me* and *me^v/me^v* mice, but that they are unable to home effectively to the thymus via the blood. We have also traced the development of B cell precursors in the bone marrow of *me/me* and *me^v/me^v* mice by detecting the B220 differentiation antigen (27–30). The results demonstrate a marked depletion of all B-lineage cells in the bone marrow of *me/me* and *me^v/me^v* mice. In addition, we have studied the development of TdT$^+$ bone marrow cells in these mutant mice. The results not only confirm the marked depletion of TdT$^+$ cells in *me/me* mice (11) and extend this observation to *me^v/me^v* mice, but show that a subset of TdT$^+$ cells that express the B220 antigen is most severely affected. Conversely, the results show that the appearance of TdT$^+$ thymocytes, which are B220$^-$, is essentially normal before the premature onset of thymic involution.

Inasmuch as the congeries of lymphoid precursor cell defects observed in this study arises from a single gene mutation, it seems likely that a common pathogenetic mechanism is implicated. The results of in vitro studies, presented in a

[1] *Abbreviations used in this paper:* cIg, cytoplasmic Ig; i.t., intrathymic; sIg, surface Ig; TdT, terminal deoxynucleotidyl transferase; TRITC, tetrarhodamine isothiocyanate.

separate paper,[2] suggest that this mechanism involves an abnormality in the stromal microenvironment of the bone marrow of *me/me* and *me^v/me^v* mice.

Materials and Methods

Animals. C3HeB/FeJ *me/me*, C57BL/6J *me/me*, C57BL/6J *me^v/me^v* mice (Ly-1.2) and their heterozygous (+/−) littermates were obtained from the colonies maintained by L. Shultz at The Jackson Laboratory, Bar Harbor, ME. Age-matched wild-type (+/+) C3HeB/FeJ mice were obtained from the Animal Resource Colonies of The Jackson Laboratory. C57BL/6 wild-type (+/+) mice (Ly-1.2) were obtained from the Mammalian Genetics and Animal Production Section of the National Cancer Institute, Frederick, MD. Congenic C57BL/6 Ly-1.1 mice were obtained from Dr. Edward Boyse, Memorial Sloan-Kettering Cancer Center, NY.

Adoptive Transfer Assays for Prothymocytes. Details of the i.v. and i.t. assay systems for prothymocytes in mice have been described previously (26). Briefly, irradiated recipients (600 rad) were injected i.v. via the tall vein or directly i.t. with bone marrow cells from congenic Ly-1-disparate donors, as reported in the text. In some experiments, C57BL/6 Ly-1.1 bone marrow cells were incubated at 4°C for 30 min with a 1:5 dilution of serum prepared from freshly clotted mouse blood (10 μl per 10^6 cells), and washed once in excess RPMI before i.v. or i.t. transfer. At various times after reconstitution, the recipients were killed and the relative and absolute numbers of donor- and host-origin thymocytes were quantified by immunofluorescence analysis for Ly-1.1 and Ly-1.2 alloantigens on the FACS (FACS IV; Becton Dickinson Immunocytometry Systems, Sunnyvale, CA).

Antisera. mAb against the Ly-1.1 and Ly-1.2 mouse pan–T cell alloantigens were obtained from New England Nuclear (Boston, MA). An affinity-purified IgG fraction of rabbit anti-TdT, prepared by Dr. F. J. Bollum (Uniformed Services University of the Health Sciences, Bethesda, MD) (31, 32), was used in these studies. Tissue culture supernatant from the 14.8 hybridoma cell line, obtained from the American Type Culture Collection (Rockville, MD), was used to label cells for the early B lineage cell surface antigen, B220 (27, 28).

Immunofluorescence. Single-cell suspensions of bone marrow and thymus were prepared in Hepes-buffered RPMI 1640 tissue culture medium (26). Cell viability was determined by exclusion of 0.1% trypan blue.

Donor- and host-origin thymocytes were detected by incubating aliquots of thymocytes in suspension with anti-Ly-1.1 or -Ly-1.2 antibodies and developing for indirect immunofluorescence with FITC-conjugated goat anti–mouse IgG (heavy and light chain–specific) (Cappel Laboratories, Cochranville, PA) (26). ≥50 × 10^4 nucleated cells from each cell suspension were analyzed on the FACS.

In some experiments, the percentage of donor-origin thymocytes that were TdT$^+$ was determined by fluorescence microscopy on cells doubly labeled for Ly-1.2 and TdT, as described previously (26).

The percentages of pre-B (cytoplasmic Ig [cIg]-positive, surface Ig [sIg]-negative) and B (sIg$^+$) cells were determined by reacting the bone marrow cells in suspension with FITC-conjugated goat anti–mouse IgG (Cappel Laboratories). Cytocentrifuge-prepared smears of labeled cells were then fixed in cold 95% ethanol/5% acetic acid. Smears were washed three times in excess PBS (pH 7.2) and developed for cytoplasmic IgM by incubation with a tetrarhodamine isothiocyanate (TRITC)-conjugated goat anti–mouse μ chain–specific antibody (affinity purified) (Southern Biotechnology, Inc., Birmingham, AL). Plasma cells were excluded from analysis on the basis of their distinctive morphology and their pattern of intense cytoplasmic immunofluorescence.

The percentage of B220$^+$ cells was determined by sequentially incubating bone marrow cell suspensions with the 14.8 mAb and with TRITC-conjugated goat anti–rat IgG (Cappel

[2] Medlock, E. S., I. Goldschneider, D. L. Greiner, and L. Shultz. Defective lymphopoiesis in the bone marrow of motheaten (*me/me*) and viable motheaten (*me^v/me^v*) mutant mice. II. Description of a microenvironment defect that inhibits the generation of terminal deoxynucleotidyl transferase-positive bone marrow cells in vitro. Manuscript submitted for publication.

Laboratories) that had been passed through a normal mouse serum Sepharose 4 B affinity column to remove crossreacting antibodies (25, 26). For TdT staining, cytocentrifuge-prepared smears of unlabeled or 14.8-labeled bone marrow and unlabeled thymocyte cell suspensions were fixed in cold absolute methanol, air dried, and incubated sequentially with 10 μl (30 μg) of rabbit anti-TdT followed by FITC-conjugated goat anti-rabbit IgG (Cappel Laboratories) (26). Singly and doubly labeled cells were counted using a Zeiss universal fluorescence microscope equipped with narrow band filters for rhodamine and fluorescein. Pre-B cells (cytoplasmic red) were distinguished from B cells (surface green); and TdT^+ cells (intranuclear green) were classified as 14.8^+ (surface red) or 14.8^-.

Determination of TdT Enzymatic Activity. The amount of TdT enzymatic activity in thymocytes was determined on a lysate obtained by a three-fold sequential freeze-thaw cycle of packed cells in a 250 mM solution of potassium phosphate. Cell debris was removed from the lysate by centrifugation at 132,000 *g*, and aliquots were incubated with a reaction mixture containing 0.2 M cacodylate buffer (pH 7.5), 0.01 mM $d(pA)_{50}$, 0.1% BSA, 8 mM $MgCl_2$, 1.0 mM 2-ME, and 1.0 mM [^{3}H]dGTP (64.3 cpm/pM) for various times at 36°C. Aliquots were applied to glass fiber squares and collected in cold 5% TCA. Filters were washed twice in 1 N HCl, rinsed twice in 95% ethanol, and air dried. The amount of radioactivity in the precipitate was determined by liquid scintillation counting. The amount of enzymatic activity contained in lysates of thymocytes was calculated from radioactivity incorporated (31). 1 U of enzyme activity is 1 nM of dGMP incorporated in 60 min.

Results

Prothymocytes. The thymocyte-regenerating capacity of bone marrow cells from *me/me* and me^v/me^v mice and their +/− littermates (Ly-1.2) was determined 14–18 d after i.v. injection into irradiated congenic C57BL/6 Ly-1.1 (+/+ wild-type) recipients. Results in Table I show that no donor-origin thymocytes were detected ($<10^6$) in the recipients of 2.5×10^6 *me/me* or 5×10^6 me^v/me^v bone marrow cells; whereas 35.8×10^6 and 8.7×10^6 donor-origin thymocytes ($Ly\text{-}1.2^+$) were detected after the i.v. transfer of 2.5×10^6 +/− littermate bone marrow cells, respectively.

Markedly different results were obtained when bone marrow cells were tested for prothymocyte activity using the i.t. adoptive transfer system. In this system, *me/me* and me^v/me^v bone marrow cells generated donor-origin thymocytes in numbers roughly equivalent to these generated by bone marrow cells from their +/− littermates (Table I). Thus, the i.t. injection of 0.15×10^6 me^v/me^v or +/− littermate bone marrow cells resulted in the appearance of 23.2×10^6 and 25.3×10^6 donor-origin thymocytes, respectively, by day 16; and 0.15×10^6 *me/me* or +/− littermate bone marrow cells generated 14.5×10^6 and 22.3×10^6 donor-origin thymocytes respectively, by day 18.

To confirm that the prothymocyte activity of me^v/me^v and +/− littermate bone marrow cells did not differ, the dose-response relationship of thymocyte regeneration was determined in the i.t. adoptive transfer system. As seen in Fig. 1, the percentage and number of donor-origin thymocytes generated at day 16 were directly proportional to the number of me^v/me^v and +/− littermate bone marrow cells that were transferred over the dose range of $0.05–0.15 \times 10^6$ cells. No differences were observed in the absolute or relative numbers of donor-origin thymocytes that were generated by the me^v/me^v and +/− littermate mice at any given cell dose. Neither were there any differences in the proportion of donor-origin thymocytes that were TdT^+ in recipients of me^v/me^v (81.8%) and +/−

TABLE I

Prothymocyte Activity of me/me and me^v/me^v Bone Marrow Cells as Detected by i.t. and i.v. Adoptive Transfer Systems

Genotype	Route of bone marrow cell injection	Number of bone marrow cells injected ($\times 10^{-6}$)	Days after bone marrow cell injection	Donor-origin thymocytes*	
				Percentage	Number ($\times 10^{-6}$) per thymus‡
me/me	i.v.	2.5	18	<1.0	<1.0
	i.t.	0.15	18	48.3 ± 15.3	14.5 ± 8.5
+/− littermate	i.v.	2.5	18	55.3	35.8
	i.t.	0.15	18	59.9 ± 9.8	22.3 ± 11.6
me^v/me^v	i.v.	5.0	14	<1.0	<1.0
	i.v.	2.5	16	<1.0	<1.0
	i.t.	0.15	16	27.3 ± 8.7	23.2 ± 9.7
+/− littermate	i.v.	5.0	14	17.6 ± 7.7	11.5 ± 6.7
	i.v.	2.5	16	9.7 ± 3.9	8.7 ± 4.3
	i.t.	0.15	16	33.1 ± 3.8	25.3 ± 12.7

4–6-wk-old congenic C57BL/6 (Ly-1.1) mice were irradiated with 600 rad and injected within 2–6 h with 4–5-wk-old C57BL/6J *me/me*, me^v/me^v, or +/− littermate bone marrow cells i.t. or i.v.

* Each point represents analysis on the FACS of ≥50,000 cells from a pool of two to four recipients, or the mean ± SD of three to four individual recipients.

‡ Determined by multiplying the total number of thymocytes in each recipient by the percentage of donor-origin ($Ly\text{-}1.2^+$) thymocytes.

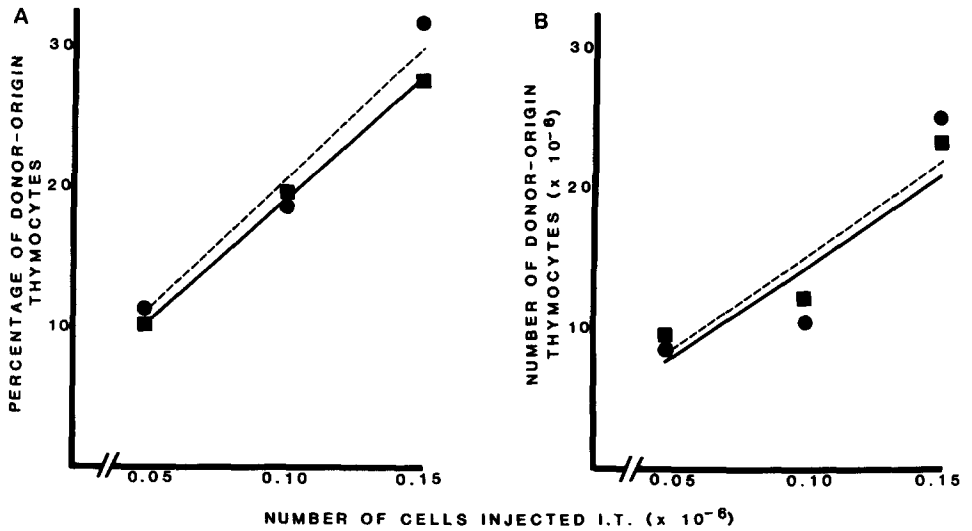

FIGURE 1. The generation of donor-origin thymocytes (Ly-1.2) as a function of dose of C57BL/6J me^v/me^v (●– – –●) or +/− littermate (■– – –■) bone marrow cells injected i.t. into irradiated (600 rad) C57BL/6 Ly-1.1 congenic recipients. (*A*) Percentage and (*B*) number of donor-origin thymocytes 16 d after i.t. injection of graded numbers of bone marrow cells. Each point represents the mean of three or four individual animals. The best fit lines were determined by linear regression analysis ($r^2 > 0.90$ in all cases).

TABLE II

Prothymocyte Activity of Normal Bone Marrow Cells after Treatment with me/me, me^v/me^v, +/− Littermate, or +/+ Wild-type Mouse Serum

Route of bone marrow cell injection	Source of serum used for treatment	Donor-origin thymocytes*	
		Percentage	Number ($\times 10^{-6}$) per thymus‡
i.v.	*me/me*	22.9 ± 14.8	11.8 ± 7.9
	+/− littermate	23.3 ± 15.3	11.4 ± 8.9
	me^v/me^v	31.6 ± 11.7	17.0 ± 11.6
	+/− littermate	28.4 ± 16.5	18.3 ± 13.6
	+/+ wild type	37.5 ± 1.3	20.6 ± 8.8
	Medium	30.5 ± 6.6	17.9 ± 5.7
i.t.	*me/me*	47.9 ± 14.2	41.7 ± 20.4
	+/− littermate	48.1 ± 17.2	43.7 ± 15.1
	me^v/me^v	33.5 ± 4.2	19.4 ± 9.3
	+/− littermate	54.7 ± 7.1	35.0 ± 8.4
	+/+ wild type	49.0 ± 14.0	41.8 ± 17.8
	Medium	40.0 ± 7.9	25.2 ± 1.2

4–6-wk-old wild-type C57BL/6 (Ly-1.2) mice were irradiated with 600 rad and injected i.v. (2.5×10^6) or i.t. (0.1×10^6) with serum-treated bone marrow cells obtained from 4-wk-old C57BL/6 (Ly-1.1) congenic mice (see Materials and Methods). Donor-origin thymocytes were quantitated by FACS analysis on day 20.

* Each value represents the mean ± SD of ≥50,000 cells from three to four individual recipients. No significant differences were observed within any of the groups ($p > 0.2$).

‡ Determined by multiplying the total number of thymocytes in each recipient by the percentage of donor-origin thymocytes.

littermate (79.6%) bone marrow cells. Furthermore, the numbers and proportions of TdT^+ and TdT^- thymocytes generated in the adoptive recipients after the i.t. injection of me^v/me^v and +/− littermate bone marrow cells were comparable to those observed after the i.t. injection of +/+ wild-type bone marrow cells (26 and our unpublished observations).

To test the possibility that autoantibodies in *me/me* and me^v/me^v mice may have prevented the generation of thymocytes in the i.v.- (but not i.t.-) injected recipients, C57BL/6 Ly-1.1 (wild-type) bone marrow cells were incubated with serum obtained from *me/me*, me^v/me^v, +/− littermate, or wild-type mice before their adoptive transfer into irradiated congenic C57BL/6 (Ly-1.2) recipients. As seen in Table II, none of the above sera significantly inhibited the generation of donor-origin thymocytes in either the i.v. or i.t. assay systems.

TdT^+ Bone Marrow Cells and Thymocytes. As determined by immunofluorescence analysis, the bone marrow of 4–5-wk-old C3HeB/FeJ *me/me* and C57BL/6J *me/me* mice was almost completely devoid of TdT^+ cells (Table III). However, in contrast to an earlier report (11), the relative and absolute numbers of TdT^+ thymocytes in two of the four C57BL/6J *me/me* mice examined were comparable to those observed in +/− littermates and wild-type C57BL/6 controls. Therefore,

TABLE III

Number of TdT$^+$ Cells in Bone Marrow and Thymus of me/me Mice

Strain	Genotype	TdT$^+$ cells*			
		Bone marrow		Thymus	
		Percentage	Number ($\times 10^{-6}$) per leg (femur and tibia)	Percentage	Number ($\times 10^{-6}$) per thymus
C3HeB/FeJ	*me/me*	0.2 ± 0.1	0.02 ± 0.02 (0.03)‡	69.8 ± 1.1§	6.5 ± 4.6 (9.1)‡
	+/− littermate	3.8 ± 0.9	0.69 ± 0.11	74.9 ± 1.5	137.6 ± 10.0
	+/+ wild type	1.8 ± 0.4	0.28 ± 0.11	77.1 ± 3.8	129.0 ± 41.4
C57BL/6J	*me/me*	0.4	0.11 (0.15)	70.8	3.7 (5.2)
	me/me	<0.1	0.02 (0.03)	75.4	96.2 (134.7)
	+/− littermate	2.5 ± 1.1	0.27 ± 0.08	77.8 ± 3.4	85.3 ± 50.0
	+/+ wild type	1.7 ± 0.7	0.35 ± 0.17	76.9 ± 1.6	90.2 ± 5.2

* Results represent the means of two mice or the mean ± SD of three to five mice.

‡ Numbers of TdT$^+$ cells in 4–5-wk-old *me/me* and +/− littermates, corrected for the reported (1) differences in body weight between *me/me* and +/− littermates.

§ Not significantly different than +/− littermate controls.

TABLE IV

Number of TdT$^+$ Cells in Bone Marrow and Thymus of me^v/me^v Mice of Various Ages

Age (wk)	Genotype	TdT$^+$ cells*			
		Bone marrow		Thymus	
		Percentage	Number ($\times 10^{-6}$) per leg (femur and tibia)	Percentage	Number ($\times 10^{-6}$) per thymus
2	*me^v/me^v*	0.4	0.02 (0.02)‡	83.1§	95.4 (114.5)‡§
	+/− littermate	2.0	0.08	80.7	16..6
3	*me^v/me^v*	0.2 ± 0.0	0.03 ± 0.01 (0.03)	75.1 ± 1.7§	37.1 ± 9.6 (44.5)§
	+/− littermate	1.7 ± 0.3	0.38 ± 0.23	74.0 ± 1.5	67.5 ± 14.2
4	*me^v/me^v*	0.5 ± 0.4	0.14 ± 0.08 (0.18)	64.6 ± 7.7§	46.7 ± 24.2 (84.1)
	+/− littermate	3.2 ± 0.6	1.23 ± 0.66	76.6 ± 5.4	130.5 ± 16.3
5	*me^v/me^v*	0.2 ± 0.2	0.02 ± 0.02 (0.03)	55.7 ± 31.9§	9.0 ± 8.1 (12.6)
	+/− littermate	1.6 ± 0.7	0.34 ± 0.14	77.3 ± 3.5	134.1 ± 71.9
6	*me^v/me^v*	0.1	0.03 (0.04)	38.6	0.4 (0.6)
	+/− littermate	1.4	0.51	75.5	110.9

* Results represent the pooled mean of two to four mice or mean ± SD of three to six mice.

‡ Numbers of TdT$^+$ cells corrected for reported (3) differences in body weight between *me^v/me^v* and +/− littermates.

§ Not significantly different than +/− littermate controls.

in order to further trace the development of TdT$^+$ bone marrow cells and thymocytes in these mutant mice, especially before the onset of thymic involution, we studied the ontogeny of these cells in C57BL/6J *me^v/me^v* mice, which have a longer life span than do their *me/me* counterparts. Like the *me/me* mice, the *me^v/me^v* mice had greatly reduced numbers and proportions of TdT$^+$ bone marrow cells at 5 and 6 wk age (Table IV). Furthermore, this deficiency was also present at 2 wk of age, even when the bone marrow cellularity was adjusted for the previously reported (3) decrease (15–25%) in body weight of *me^v/me^v* mice in relation to their +/− littermates. However, the relative and absolute number of TdT$^+$ thymocytes, when adjusted for the reported differences in body weight (3), approached normal values in 2- and 3-wk-old *me^v/me^v* mice, and then decreased rapidly with the early onset of thymic involution.

TABLE V

TdT Enzymatic Activity of me/me and me^v/me^v Thymocytes

Genotype	TdT^+ cells (%)	TdT (U) per 10^8 TdT^+ thymocytes*
me/me	73.7	19.3
+/− littermate	81.2	22.0
me^v/me^v	67.4 ± 4.9	24.3 ± 2.2
+/− littermate	76.5 ± 4.7	21.2 ± 4.3
+/+ wild type	79.2 ± 1.5	16.9 ± 0.5

Thymocytes were obtained from 4–5-wk-old C57BL/6J *me/me*, C57BL/6J me^v/me^v, their +/− littermates, and C57BL/6 +/+ wild type mice. Results represent the means of one to three mice or the mean ± SD of four to six mice. No significant differences were observed between groups.

* The amount of TdT enzymatic activity was determined on a lysate of packed thymocytes (see Materials and Methods). The number of TdT units per TdT^+ thymocyte was determined by dividing the total amount of TdT enzymatic activity per cell by the percentage of TdT^+ thymocytes.

TABLE VI

B-lineage Cells in Bone Marrow of me/me and me^v/me^v Mice

Strain	Genotype	Total nucleated ($\times 10^{-6}$) cells per leg (femur and tibia)	Distribution of B-lineage cells (%)				
			TdT^+		$B220^+$	cIg^+, sIg^- (pre-B cells)	sIg^+ (B cells)
			$B220^+$	$B220^-$			
C3HeB/FeJ	*me/me*	9.5 ± 4.2	ND	ND	1.3 ± 0.3	0.9 ± 0.4	1.4 ± 0.6
	+/− littermate	18.3 ± 1.4	ND	ND	16.6 ± 5.8	6.0 ± 2.1	12.5 ± 1.9
	+/+ wild type	15.7 ± 4.3	51.6 ± 8.9	48.4 ± 10.4	18.7 ± 2.4	6.6 ± 0.9	10.0 ± 2.7
C57BL/6J	me^v/me^v	21.8 ± 5.2*	7.1 ± 6.9	92.9 ± 8.6	2.4 ± 0.2	1.0 ± 0.2	1.8 ± 0.5
	+/− littermate	21.8 ± 4.3	58.4 ± 8.7	41.6 ± 10.7	20.2 ± 4.9	7.8 ± 0.6	10.0 ± 2.0
	+/+ wild type	20.8 ± 2.9	61.4 ± 8.0	38.6 ± 9.2	21.1 ± 5.1	7.6 ± 0.8	11.2 ± 0.8

Bone marrow cells from 4–5-wk-old mice were stained with anti-TdT, anti-IgM, anti-IgG and/or anti-B220 (14.8) antibodies (see Materials and Methods). Results represent the means ± SD of three to four mice.

* Not significantly different from +/− littermate controls.

To confirm the immunofluorescence data, we determined the enzymatic activity of the TdT in thymocytes from *me/me* and me^v/me^v mice. As seen in Table V, the amount of enzymatic activity per TdT^+ thymocyte in *me/me* and me^v/me^v mice did not differ significantly from that observed in +/− littermates or in +/+ wild-type, age-matched controls.

B-lineage Bone Marrow Cells. Bone marrow cells from *me/me* and me^v/me^v mice were examined by immunofluorescence for abnormalities in the development of early B lineage cells. The percentages and absolute numbers of B cells (sIg^+), pre-B cells (cIg^+, sIg^-) and $B220^+$ cells (presumptive total B lineage cells) in the bone marrow of *me/me* and me^v/me^v mice were significantly decreased (from 85.0% to 93.0%) with respect to their counterparts in +/− littermates and in +/+ wild-type controls (Table VI). In addition, <10% of the TdT^+ bone marrow cells in *me/me* and me^v/me^v mice expressed the B220 antigen; whereas approxi-

mately half of the TdT^+ bone marrow cells in +/− littermate and +/+ wild-type mice were $B220^+$.

Discussion

We used a newly developed i.t. adoptive transfer system that is independent of cell migration to show that the bone marrow of *me/me* and me^v/me^v mice contains apparently normal numbers of prothymocytes. Preliminary experiments, confirmed here, found that *me/me* and me^v/me^v bone marrow cells are unable to repopulate the thymus of irradiated recipients after i.v. transfer, thereby suggesting that these mutant mice lacked prothymocytes. However, when these bone marrow cells are injected directly into the thymus, they are able to generate thymocytes as efficiently as can their counterparts from normal mice. Hence, although not formally confirmed by cell tracer studies (33, 34), our results suggest that *me/me* and me^v/me^v prothymocytes fail to generate thymocytes in the i.v. transfer system because they are unable to migrate to or successfully enter the thymus.

Although developmental abnormalities of prothymocytes appear to be important predisposing factors to autoimmunity in several mutant mouse strains (20–24), to date, only the prothymocytes in *me/me* and me^v/me^v mice appear to have a thymus homing defect. In contrast, prothymocytes from the New Zealand, BXSB, and MRL models of systemic lupus erythematosus and the SJL/J murine model of experimental allergic encephalomyelitis readily generate thymocytes in the i.v. adoptive transfer system (20–24), and their bone marrow contains normal or elevated numbers of TdT^+ cells.

The lack of detectable prothymocyte activity in *me/me* and me^v/me^v mice in the i.v. assay does not appear to be due to the presence of autoantibodies, inasmuch as pretreatment of normal bone marrow cells with serum from these mutant mice fails to inhibit their thymocyte regenerative capability in either the i.v. or i.t. assay. In addition, no evidence for inhibitory cell activity has been found in preliminary mixing experiments of me^v/me^v and normal strain bone marrow cells (K. Komschlies, unpublished observations). Although the nature of the observed migratory defect is unknown, it is clear that the thymus is not at fault in these mutant mice. Thus, +/− littermate or congenic +/+ wild-type prothymocytes injected i.v. into *me/me* and me^v/me^v mice home efficiently to the thymus (L. Shultz, unpublished observations). The results of these latter experiments also speak against a possible role for autoantibodies against prothymocytes.

It is tempting to speculate that the homing defect detected in the i.v. assay for prothymocytes is a major contributing factor to the early onset of thymic involution in *me/me* and me^v/me^v mice (2, 3). However, if prothymocytes lack the ability to home effectively to the thymus, what would account for the initial colonization of the thymus in these mutant mice and the appearance of thymocytes with rearranged T cell receptor β genes (35)? And what would account for the presence of relatively normal numbers of peripheral T cells in juvenile mice (2, 3)? One plausible scenario is that only one wave of colonization of the thymus by prothymocytes occurs during fetal life in *me/me* and me^v/me^v mice. Inasmuch as the generative cycle of intrathymic progenitors is ~30 d (26, 36), failure to replace these progenitors by subsequent waves of prothymocytes would result in

the onset of thymic involution at 2–3 wk of age, which corresponds very closely to the situation in *me/me* and *me^v/me^v* mice. However, peripheral T cells, being mostly long-lived, would persist well beyond the onset of thymic involution. Thus, it has been observed that in normal mice (*a*) multiple waves of thymus colonization by prothymocytes occur during fetal development (F. Jotereau, personal communication); (*b*) prothymocytes seed to the fetal thymus by a different, partly nonvascular, route than to the postnatal thymus (37, 38); (*c*) the thymus is more permeable to the passage of cells in fetal and neonatal life than in adult life (39, 40); (*d*) prothymocytes originate primarily in the liver of fetal mice, and in the bone marrow of postnatal mice (25, 26, 37, 41); (*e*) thymocytes in the fetal and neonatal periods have different properties than those that appear in older animals (42–44); and (*f*) thymectomy conducted in 3–4-wk-old mice does not significantly affect the levels of peripheral T cells until several months thereafter (45). Studies are in progress to determine whether defective homing is also exhibited by fetal prothymocytes in *me/me* and *me^v/me^v* mice, or only by prothymocytes that develop after the primary colonization of the thymus has occurred.

It could, of course, be argued that the explanation for the present results is that the i.v. and i.t. assays detect different lineages of bone marrow prothymocytes, and that one of these lineages may be missing in *me/me* and *me^v/me^v* mice. However, no differences between the i.v. and i.t. assays have been found in experiments in which normal mice were used to create radiation chimeras. In such chimeras, the ratios of donor-origin TdT$^+$ and TdT$^-$ thymocytes and of peripheral T cell subsets with helper and suppressor antigenic phenotypes approximated normal values in both assay systems (25, 26). Similarly, in the present study, prothymocytes from *me/me* and *me^v/me^v* mice generated normal ratios of TdT$^+$ (presumptive cortical) and TdT$^-$ (presumptive medullary) thymocytes in the i.t. assay.

A more likely explanation is that the i.v. and i.t. assays detect prothymocytes at different stages of maturation; the i.v. assay detecting mature (migratory) prothymocytes only, and the i.t. assay detecting both mature and immature (nonmigratory) prothymocytes. Given that the prothymocyte activity of *me/me* and *me^v/me^v* bone marrow, as detected by the i.t. assay, was comparable quantitatively to that of +/− littermate bone marrow, this hypothesis would predict that almost all of the prothymocytes in these mutant mice belong to the immature cell subset. Evidence supporting this conclusion is provided by in vitro studies[2] of the development of TdT$^+$ bone marrow cells in *me/me* and *me^v/me^v* mice. The results show that, although bone marrow from these mutant mice is markedly deficient in TdT$^+$ cells, it can readily generate such cells in vitro when placed on a feeder layer of stromal cells from +/+ wild-type bone marrow. However, unlike bone marrow from normal mice, only the B220$^-$ subset of TdT$^+$ cells (presumptive T lineage, see below) is generated in vitro by *me/me* and *me^v/me^v* bone marrow cells. These findings therefore suggest that the mutant prothymocytes undergo a maturational arrest at the stage immediately preceding the expression of TdT (i.e., at the "pre-TdT-cell" stage [14]).

In addition to the defective development of prothymocytes, the results of this study show that there is a marked deficiency of all of the identifiable members

of the B lymphocyte series in the bone marrow of *me/me* and *me^v/me^v* mice, including pre-B cells and their B220$^+$, cIg$^-$, sIg$^-$ precursors. Thus it is possible that the depletion of peripheral B lymphocytes that has been observed in *me/me* and *me^v/me^v* mice (3–8) may result from the defective development of early B cell precursors. Alternatively, the deficiency of early B lineage precursors and peripheral B cells may be due to the rapid exit of the former cells from the bone marrow, followed by the rapid differentiation of the latter cells into plasma cells (46, 47).

The levels of pre-B cells that we observed in the bone marrow of 4–5-wk-old *me/me* (0.97%) and *me^v/me^v* (1.0%) mice approximate those recently reported by McCoy et al. (48) for 19–22-d-old *me/me* mice (2.1%). However, the levels of pre-B cells that we detected in the age-matched +/− littermates and +/+ wild-type controls (6.0–7.8%) were substantially higher than those reported by McCoy et al. (47), and were comparable instead to previously reported values for normal adult mice (48, 49). Thus, our results indicate that young adult *me/me* and *me^v/me^v* mice are markedly depleted of early B lineage bone marrow cells, whereas results of McCoy et al. (47) suggest that 3-wk-old mutant mice are not significantly depleted of B cell precursors. These observations are consistent with previous reports of a marked decrease in the numbers of peripheral B cells in *me/me* and *me^v/me^v* mice, which first becomes apparent after 3 wk of age. It appears therefore that the generation of B cells, like that of thymocytes, is relatively normal in the neonatal period, but is markedly restricted thereafter by an arrest in the maturation of lymphoid progenitor cells. This conclusion is further supported by our observation that B220$^+$ bone marrow cells, which include all of the pre-B cells and their immediate precursors (27, 28), also are severely depleted in young adult *me/me* and *me^v/me^v* mice. It is important to note that the B220 antigen is absent from prothymocytes,[3] pluripotent hemopoietic stem cells (27, 28), and myeloid and erythroid progenitor cells (27, 28). It is also important to note that some peripheral B cells are present in *me/me* and *me^v/me^v* mice (3–8, 47); and that, in the presence of elevated levels of compensatory B cell maturation factors (BMF), they may account for the reported polyclonal B cell hyperactivity and autoantibody production in these animals (5, 9, 10).

As reported previously (11) and confirmed here, marked deficiencies are apparent in the generation of TdT$^+$ bone marrow cells in *me/me* mice. We now provide evidence that this deficiency occurs in *me^v/me^v* mice as well. Moreover, we show that TdT$^+$ bone marrow cells can be divided into two roughly equal subsets on the basis of their differential expression of the B220 antigen, and that *me^v/me^v* mice are most severely depleted of the B220$^+$ subset. Thus, the deficiency of the B220$^+$ subset of TdT$^+$ bone marrow cells in *me/me* and *me^v/me^v* mice correlates with the deficiency of early B-lineage cells; the ability to generate the B220$^-$ subset of TdT$^+$ cells correlates with the ability to generate prothymocytes.[2] In support of this notion, we have recently shown[2] that all prothymocyte activity measured by both the i.t. and i.v. adoptive transfer systems is found in the B220$^-$ fraction of normal mouse bone marrow cells; others have shown that

[3] Greiner, D. L., E. S. Medlock, and I. Goldschneider. Separation of murine lymphopoietic precursor cell subsets on the fluorescence-activated cell sorter using the 14.8 monoclonal antibody to the B220 antigen. Manuscript in preparation.

all B cell precursor activity detected by the in vitro clonable B cell assay is present in the $B220^+$ fraction of bone marrow cells (27, 28).

It is unlikely that early lymphoid precursor cells in *me/me* and me^v/me^v mice are depleted (or their development is inhibited) by autoantibodies, inasmuch as we (unpublished observations) and others (11) have been unable to detect antibodies in the serum of these mice to bone marrow TdT^+ cells or to prothymocytes. It is also unlikely that the defective development of lymphoid precursors in *me/me* and me^v/me^v mice is due to stress-induced increases in the levels of circulating adrenal corticosteroid hormones (50–52), because TdT^+ bone marrow cells are markedly depleted in 2- and 3-wk-old me^v/me^v mice, at a time when the number of steroid-sensitive TdT^+ thymocytes (52) is essentially normal. Finally, it is unlikely that lymphopoiesis occurs at a site other than the bone marrow in *me/me* and me^v/me^v mice. Neither TdT^+ cells nor pre-B cells are present in significant numbers in the spleen (11, 47, and our unpublished observations), and the onset of thymic involution and depletion of peripheral B cells in these mutant mice significantly precedes that in the +/− littermate and +/+ wild-type controls.

While the defective development in *me/me* and me^v/me^v mice of prothymocytes, B cell progenitors, and TdT^+ cells appears to be indigenous to the bone marrow and results from a single gene mutation, it is likely that a common mechanism is implicated. We believe that the most likely explanation for the observed defects in the development of lymphopoietic progenitor (or stem) cells in *me/me* and me^v/me^v mice is an abnormality in the microenvironment of the bone marrow. Thus, in a separate paper,[2] we have used a selective culture system for TdT^+ cells (53) to demonstrate that stromal cell feeder layers generated by bone marrow cells from *me/me* and me^v/me^v mice not only fail to support the generation of xenogeneic TdT^+ cells in vitro, but prevent feeder layers of stromal cells from +/− littermate bone marrow from doing so. Furthermore, although *me/me* and me^v/me^v bone marrow cells can generate TdT^+ cells when placed onto stromal cell feeder layers from normal mice, only the $B220^-$ subset of TdT^+ cells is effectively generated. These in vitro results appear to correlate with our in vivo findings, in which the $B220^+$ (presumptive B lineage) subset of TdT^+ bone marrow cells is almost totally depleted in *me/me* and me^v/me^v mice, and in which prothymocytes appear to be developmentally arrested at the pre-TdT^+-cell stage (14).

Summary

This study identifies defects in the early stages of lymphopoiesis that may contribute to the abnormalities in the development and/or function of peripheral T and B lymphocytes in mice homozygous for the motheaten (*me/me*) and viable motheaten (me^v/me^v) mutations. The results indicate that in *me/me* and me^v/me^v mice (*a*) prothymocytes in bone marrow are present in essentially normal numbers, as determined by intrathymic injection, but apparently lack the ability to home effectively to the thymus, as determined by intravenous transfer; (*b*) early B lineage cells in bone marrow, identified by the B220 antigen, are markedly depleted, including immature B cells (sIg^+), pre-B cells (cIg^+, sIg^-), and pro-B cells ($B220^+$, cIg^-, sIg^-); (*c*) TdT^+ bone marrow cells, especially a subset that

expresses the B220 B lineage antigen, are markedly depleted by two weeks of age; (*d*) normal numbers of TdT^+ thymocytes are present during the first 3 wk of postnatal life, but rapidly decrease thereafter. The results further indicate that neither the defective thymus homing capacity of prothymocytes nor the deficiency of TdT^+ bone marrow cells is due to autoantibodies.

The possible relationship of the defective development of lymphoid precursor cells to the premature onset of thymic involution and to the abnormalities of peripheral T and B lymphocytes in *me/me* and me^v/me^v mice is discussed; as are the results of in vitro studies (presented in a companion paper), which suggest that a primary defect in the stromal microenvironment of the bone marrow is responsible for the abnormal development of the lymphoid precursor cells.

We thank Mrs. Frances Tausche, Ms. Marianne Angelillo, and Mr. Jerome Murphy for their expert technical assistance, and Mrs. Ruth Conrod for typing the manuscript.

Received for publication 23 April 1986.

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