INFLUENCE OF THYMIC GENOTYPE ON THE SYSTEMIC LUPUS ERYTHEMATOSUS-LIKE DISEASE AND T CELL PROLIFERATION OF MRL/Mp-*lpr/lpr* MICE*

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The availability of inbred murine strains (NZB, NZB/W, BXSB, MRL) that spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus (SLE),[1] enables one to study the preclinical stage of this disorder, to discern possible common etiologic pathways for its development among these strains, and to manipulate them experimentally so as to determine their essential immunologic abnormalities. In terms of evaluating the latter abnormalities, MRL and BXSB mice (1) are preferable to the NZB/W strain in that they express commonplace histocompatibility types (NZB/W, $H\text{-}2^{d+z}$; MRL, $H\text{-}2^{k}$; BXSB, $H\text{-}2^{b}$) and offer both acute severe and chronic late-life forms of SLE within each strain (1–3). Thereby, transplantation of various organs and cell types between animals with acute and late-life SLE is possible unimpeded by antigenic differences between graft and host.

Histopathologically, all of the immunologically abnormal strains cited above exhibit early thymic atrophy, most severe in the cortex, but also involving the medulla (2). This finding, as well as thymic hormonal defects (4, 5) and premature thymic involution with prominent degeneration and vacuolization of epithelial cells (6, 7) observed in New Zealand (NZ) mice, suggests a role for the thymus in the pathogenesis of murine SLE. Nevertheless, until recently, no definitive experiments have been performed to substantiate the postulated role of the thymus in the disease of this strain or in murine SLE in general.

In the present studies, we have exchanged thymic transplants between the congenic MRL/Mp-*lpr/lpr* and MRL/Mp-+/+ substrains to clarify whether inherent thymic defects contribute to the early appearance of severe SLE-like disease and massive T cell proliferation in the former (50% mortality in males and females at ~5–6 mo) but not in the latter substrain (50% mortality at 17–23 mo for female and males, respectively). Our results indicate that, although a thymus is necessary for the lymphoid hyperplasia and early SLE of *lpr/lpr* mice, the thymic genotype is irrelevent because the abnormal immunologic characteristics develop regardless of whether the

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[1] *Abbreviations used in this paper:* BUN, blood urea nitrogen; Con A, concanavalin A; dsDNA, double-stranded DNA; NZ, New Zealand; sIg, surface Ig; SLE, systemic lupus erythematosus; SRBC, sheep erythrocytes; ssDNA, single-stranded DNA; TLI, total lymphoid irradiation.

thymus is of *lpr/lpr* or +/+ genotype. Conversely, a thymus of the *lpr/lpr* genotype does not confer early, severe SLE or lymphoproliferation to the +/+ mouse.

Materials and Methods

Mice. MRL/Mp-*lpr/lpr* and MRL/Mp-+/+ mice originally developed by Murphy and Roths (1) were bred and maintained at the Scripps Mouse Colony, Scripps Clinic and Research Foundation, La Jolla , Calif. All mice used in these experiments were females. Experimental and control animals were housed identically and allowed standard laboratory food and water ad lib. Mice were followed daily for signs of disease, lymphadenopathy, and survival and were bled monthly by retroorbital sinus puncture for serologic examinations.

Thymectomy. The following five groups of mice were studied: (*a*) MRL/Mp-*lpr/lpr* mice thymectomized when newborn (1-d old) and not transplanted with thymus; (*b*) MRL/Mp-*lpr/lpr* mice thymectomized when newborn and transplanted at 1 mo of age with MRL/Mp-+/+ thymus; (*c*) MRL/Mp-+/+ mice thymectomized when newborn and transplanted at 1 mo of age with MRL/Mp-*lpr/lpr* thymus; (*d*) control unmanipulated MRL/Mp-*lpr/lpr*; and (*e*) control, unmanipulated MRL/Mp-+/+ mice. Thymectomy was performed through a small longitudinal incision over the sternum, and the thymus was withdrawn by a wire loop as described previously (8). In transplanted animals, a whole thymus from a 1-mo old donor was grafted under the skin at the back of the neck of the recipient by using a 19-gauge needle (8). Apart from histologic examinations, the absence or presence of a thymus in thymectomized and thymus-transplanted animals was judged at 3 mo of age by determining the frequency of Thy-1.2$^+$ cells in peripheral blood, number of plaque-forming cells in spleens, and the titer of hemagglutinating antibodies 5 d after intravenous immunization with 2×10^8 sheep erythrocytes (SRBC)—a T dependent antigen. Additionally, proliferative responses to the T cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) were examined in splenocytes of five mice from each of these groups. Representative results are shown in Table I. Mice that were considered incompletely thymectomized or not reconstituted (~20%) were discarded and not included in the results given below.

Histologic Studies. Unless otherwise indicated, mice were killed when moribund and then autopsied. Sections of thymuses, spleens, mesenteric and peripheral lymph nodes, and kidneys were fixed in Bouin's fluid and stained with hematoxylin and eosin and periodic acid-Schiff stain. Severity of glomerulonephritis was graded from 0 to 4$^+$ as described previously (2).

Serologic Studies. Blood urea nitrogen (BUN) was determined by Azostix strips as recommended by the manufacturer (Ames Division, Miles Laboratories, Inc., Elkhart, Ind.). IgG was estimated by single radial immunodiffusion (9). Anti-single-strand DNA (ssDNA) and anti-double-strand DNA (dsDNA) antibodies were measured by a modified Farr assay as described (10). The modified Raji cell assay (2) was used for detecting immune complexes in murine sera. Serum retroviral envelope gp70 was measured by a radioimmunoassay (11).

Cellular Analyses. Spleen and lymph node cell preparations, total spleen and lymph node cell counting, cell viability determinations, and assessment of surface-Ig-bearing and Thy-1.2

TABLE I

*Mitogenic Stimulation and Responses to SRBC In Thymectomized and Thymus-transplanted MRL Mice**

	Thy-1.2$^+$	Con A	PHA	SRBC PFC‡/10^6 cells	SRBC-agglutination titer
	%	*stimulation index*			
lpr/lpr (unmanipulated	67	7.5	4.2	237	32
+/+ (unmanipulated)	59	17.3	7.0	567	128
lpr/lpr (no implant)	9	1.7	1.2	13	2
+/+ thymus → *lpr/lpr* Tx§	51	4.9	3.7	117	16
lpr/lpr thymus → +/+ Tx	41	8.6	5.2	421	64

* Animals were tested at 3 mo of age.

‡ PFC, plaque-forming cells.

§ Tx, thymectomized; →, transplanted into.

alloantigen-bearing cells were done as before (12, 13). Proliferative responses to Con A and PHA were assessed after incubation for 3 d of triplicate cultures containing 2×10^5 splenocytes in microtiter plates with 0.25 μg and 100 μl each of the respective mitogens per culture; 0.5 μCi of [^{3}H]thymidine was added 18 h before harvesting. The frequency of polyclonal Ig-secreting cells in splenocyte populations was determined by a reverse hemolytic plaque assay in which SRBC coated with rabbit anti-mouse IgM,κ were developed with a rabbit anti-mouse Ig and guinea pig complement (14).

Results

Survival Rate. The median survival times (Fig. 1) of unmanipulated MRL/Mp-*lpr/lpr* and MRL/Mp-+/+ mice were 160 and 510 d, respectively. Of these control *lpr/lpr* mice, 100% developed grossly apparent lymphoid hyperplasia and dermatitis as previously described (1–3). The lymphoid hyperplasia became evident clinically when the animals were ~3.5 mo of age and progressed thereafter until death. The *lpr/lpr* mice thymectomized and subsequently transplanted with +/+ thymuses retained the disease phenotype of the unmanipulated *lpr/lpr* mice, including lymph node hyperplasia and a 50% mortality at 186 d. The +/+ mice were similarly unchanged by thymectomy and transplation with *lpr/lpr* thymuses; these mice did not develop lymphoid hyperplasia and 50% remained alive at 498 d of age, which is not appreciably different than that of the unmanipulated controls. In contrast, *lpr/lpr* mice successfully thymectomized when newborn but not transplanted with thymuses did not develop lymph node hyperplasia or dermatitis, and 100% of them were alive by the 13 mo of age, a point well beyond the 90% death rate of control, unmanipulated

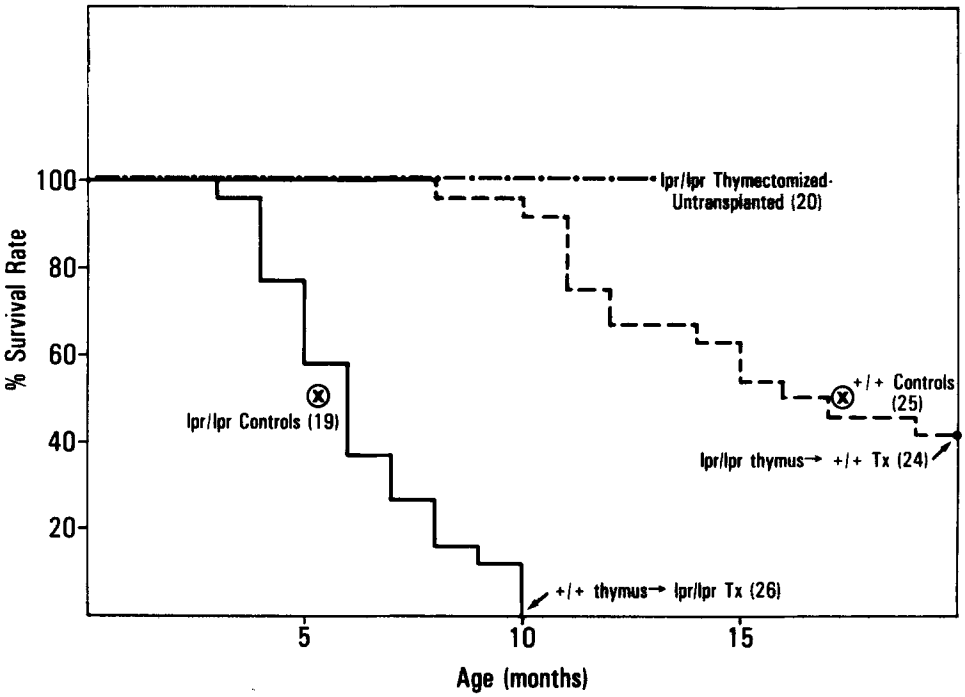

FIG. 1. Survival rates of thymectomized and of thymus-transplanted MRL mice. Numbers in parentheses indicate the numbers of mice studied in each group.

lpr/lpr mice (9 mo of age). Of a total of 20 thymectomized, untransplanted animals, 10 mice in apparently good health and without lymphoid hyperplasia were killed at 13 mo of age for histologic and cellular studies (see below). The remaining 10 animals are still alive and under observation.

Histolic Observations. About 90% (Table II) of control, unmanipulated *lpr/lpr* mice had severe glomerulonephritis by the 5th mo. Similarly, ~80% of *lpr/lpr* mice thymectomized and transplanted with +/+ thymus developed severe glomerulonephritis by 5 mo. In contrast, only 10% of the thymectomized, untransplanted *lpr/lpr* mice that were killed at 13 mo of age had developed severe glomerulonephritis. Approximately 30–40% of the +/+ thymectomized mice transplanted with *lpr/lpr* thymus had severe glomerulonephritis at the time of death (12–24 mo old). Of the remaining animals one-half died of pneumonia without glomerulonephritis, and in the other one-half the cause was undetermined.

Histologically, hyperplasia and proliferation were evident in the lymph nodes of all unmanipulated as well as thymectomized and +/+ thymus-transplanted *lpr/lpr* mice, but in none of the successfully thymectomized untransplanted animals. Identically to unmanipulated +/+ mice, +/+ mice thymectomized and transplanted with *lpr/lpr* thymus had little or no lymphoid hyperplasia at the time of death.

Serologic Characteristics. Serologically, the *lpr/lpr* recipients of +/+ thymus and the +/+ recipients of *lpr/lpr* thymus behaved like the unmanipulated respective groups; hypergammaglobulinemia, high levels of anti-ssDNA and anti-dsDNA antibodies, retroviral gp70, immune complexes, and BUN were observed in the former, but not the latter recipients (Table III). However, thymectomized, untransplanted *lpr/lpr* mice had significantly reduced levels in each of these categories compared with unmanipulated controls ($P < 0.001$, Student's t test).

Cellular Characteristics. As indicated above, thymectomized, untransplanted *lpr/lpr* mice did not develop the characteristic lymph node hyperplasia observed upon gross examination of their 4–5-mo old unmanipulated counterparts. Approximately 6×10^8–7×10^8 cells were recoverable from mesenteric lymph nodes of a diseased 5-mo-old unmanipulated *lpr/lpr* mouse or an *lpr/lpr* mouse thymectomized and transplanted with +/+ thymus, and ~90% of these cells were Thy-1.2 antigen positive (Table IV). In comparison, successfully thymectomized, untransplanted *lpr/lpr* mice that were killed had mesenteric, inguinal, and submaxillary nodes similar in size to those in

TABLE II

Relationship between the Thymus and Glomerulonephritis in MRL Mice

	Degree of glomerulonephritis				
	0	1+	2+	3+	4+
			%		
lpr/lpr (unmanipulated) (10)*	0	0	10.0	50	40
+/+ thymus → *lpr/lpr* Tx‡ (24)	8.3	12.5	0	37.5	41.6
lpr/lpr thymus → +/+ Tx (10)	30.0‖	20.0‖	20.0	20.0	10.0
lpr/lpr Tx (no implant) (10)§	60.0	20.0	10.0	0	10.0

* Numbers in parentheses indicate the numbers of mice from which kidney biopsies were available.
‡ Tx, thymectomized; →, transplanted into.
§ Successfully thymectomized *lpr/lpr* mice that were experimentally killed at 13 mo of age.
‖ +/+ mice without glomerulonephritis that died relatively early had developed pneumonia.

TABLE III

*Relationship between the Thymus and Serologic Characteristics of MRL Mice**

	IgG	α-ssDNA	α-dsDNA	Immune complexes	gp70	BUN
	mg/ml	*% binding*		*mg/ml*	*μg/ml*	*mg/dl*
lpr/lpr (unmanipulated)	31.0 ± 5.0§	77.8 ± 10.2	28.6 ± 5.8	2,935.5 ± 793.5	27.1 ± 38.1	51.1 ± 9.6
+/+ (unmanipulated)	13.4 ± 0.9	51.7 ± 13.0	12.5 ± 3.6	67.2 ± 32.0	10.7 ± 2.8	27.0 ± 10.0
lpr/lpr thymus → +/+ Tx‡	10.8 ± 1.2	54.6 ± 14.1	15.3 ± 8.2	142.0 ± 87.8	9.5 ± 6.2	22.2 ± 5.1
+/+ thymus → *lpr/lpr* Tx	30.7 ± 8.2	82.2 ± 17.8	36.7 ± 18.3	2,352.1 ± 1,310.2	20.4 ± 8.7	51.0 ± 12.4
lpr/lpr Tx (no implant)	11.6 ± 3.1	23.8 ± 14.8	10.4 ± 3.4	119.0 ± 79.6	7.7 ± 6.5	27.0 ± 8.2

* Sera obtained at 5 mo of age.

‡ Tx, thymectomized; →, transplanted into.

§ Levels of IgG, anti (α)-ssDNA, α-dsDNA, immune complexes, gp70, and BUN were significantly different (Student's *t* test, $P < 0.001$) in thymectomized, untransplanted *lpr/lpr* mice compared with unmanipulated controls. All other experimental groups were not significantly different from their respective controls.

TABLE IV

*Relationship between the Thymus and T Cell Proliferation in Lymphoid Organs of MRL Mice**

	No. cells/MLN‡	No. cells/ spleen	Splenocytes			
			Thy-1.2$^+$	sIg$^+$	Thy-1.2$^+$	sIg$^+$
	× 10^{-6}		%		× 10^{-6}	
lpr/lpr § (unmanipulated)	679 ± 113 (91% Thy-1.2$^+$)	287 ± 45	67 ± 8	27 ± 5.0	192	77
+/+‖ (unmanipulated)	ND**	81 ± 17	36 ± 5	51 ± 13	29	41
lpr/lpr thymus → +/+ Tx§‖	ND	110 ± 11	40 ± 9	52 ± 9	44	57
+/+ thymus§ → *lpr/lpr* Tx¶	590 ± 210 (87% Thy-1.2$^+$)	310 ± 59	74 ± 11	19 ± 4	229	58
lpr/lpr Tx (no implant)‖	13 ± 8	109 ± 23	11 ± 3	67 ± 9	12	73

* Results from groups of five animals.

‡ MLN, mesenteric lymph node;

§ Tx, thymectomized; →, transplanted into.

‖ 13 mo old.

¶ 5 mo old.

** Not done.

immunologically normal murine strains. That is, only 13 × 10^6 cells were recovered from isolated mesenteric nodes of such animals. Similarly, thymectomized, untransplanted *lpr/lpr* mice did not have the splenomegaly so commonly found in unmanipulated or +/+ thymus-transplanted 4–5-mo-old *lpr/lpr* animals. As shown in Table IV, approximately threefold more mononuclear cells were recovered from spleens of unmanipulated or +/+-thymus transplanted mice than from thymectomized, untransplanted *lpr/lpr* mice. Moreover, in the splenocyte populations from thymectomized and, subsequently, +/+ thymus-transplanted as well as in unmanipulated *lpr/lpr* mice, ~70% of cells were positive for Thy-1.2 antigen compared with only 11% in thymectomized, untransplanted mice. Conversely, +/+ thymus-transplanted or unmanipulated *lpr/lpr* mice had 19–27% of surface Ig (sIg)-positive cells in their spleens, whereas thymectomized, untransplanted animals had 67% of similarly stained cells. Therefore, in absolute numbers, the spleens and mesenteric lymph nodes of unmanipulated or +/+ thymus-transplanted *lpr/lpr* mice had ~80- to 90-fold more T cells than those of thymectomized, untransplanted *lpr/lpr* mice. In addition, these thymectomized, untransplanted animals had ~10-fold less spontaneous Ig-secreting cells in their spleens than unmanipulated or +/+ thymus transplanted *lpr/lpr* mice (Table

TABLE V

*Effect of Thymectomy on the Frequency of Spontaneous Ig-secreting Splenocytes of lpr/lpr mice**

	No. IgSC/10^6 splenocytes‡	Total/spleen
lpr/lpr (unmanipulated)§	12,373 ± 674	3,551,051
lpr/lpr Tx‖ (no implant)¶	2,998 ± 196	326,782

* Results from groups of five animals (mean ± SD).
‡ IgSC, Ig-secreting splenocytes.
§ 5 mo old.
‖ Tx, thymectomized.
¶ 13 mo old.

V). There were no appreciable differences in numbers of either total spleen and lymph node cells or Thy-1.2 and sIg-bearing cells between unmanipulated or thymectomized, *lpr/lpr* thymus-transplanted +/+ mice.

Discussion

The important and novel point made by these studies pertains to the irrelevance of the thymus genotype to the phenotype of autoimmune disease in the MRL strain of mice. Our studies demonstrate that the thymus must be present for expression of lymphoid hyperplasia caused by T cell proliferation and for early SLE in the *lpr/lpr* phenotype of MRL/Mp mice but the genotype of the thymus is irrelevant. Conversely, these experiments demonstrate that differentiation of +/+ stem cells (from the congenic substrain) to T cells under the hormonal or microenvironmental influence of a thymus that possessess the *lpr* genotype does not lead to abnormal T cell differentiation and early autoimmunity. In addition, because early thymectomy improved survival of *lpr/lpr* mice, we may conclude that the B cell hyperactivity leading to the immune complex-mediated aspects of SLE was dependent upon the presence of a thymus and not exclusively caused by either an intrinsic B cell defect or mitogenic stimulation that causes polyclonal B cell activation. Our experiments have not established whether the T cell defect associated with the disease is acquired at the prethymic or thymic level. Nor have they determined whether the thymic effects necessary for expression of T cell proliferation are exerted within the thymic microenvironment or extrathymically via thymic hormones (15), or both. Nevertheless these experiments certainly exclude causation of the disease by intrinsic abnormalities of the *lpr/lpr* thymus (hormonal or microenvironmental) because the *lpr/lpr* early, severe SLE is not prevented or delayed by +/+ thymus and transplantation of *lpr/lpr* thymus to the +/+ mice does not cause T cell proliferation and early SLE in this substrain. After our initial report (16) and during the completion of our studies, others also reported inhibition of lymphoid hyperplasia and early disease development in neonatally thymectomized *lpr/lpr* mice (17).

Several other observations indicate the importance of the *lpr* gene and of associated T cell proliferation in the development of early, severe SLE in MRL mice. We previously demonstrated that total lymphoid irradiation (TLI) or whole body irradiation of 3-mo-old *lpr/lpr* mice inhibited expression of lymphoid hyperplasia and the early appearance of disease (18). Of interest, the beneficial effect of TLI was seen

whether or not the thymus was shielded during irradiation (A. N. Theofilopoulos, B. L. Kotzin, and S. Strober, unpublished observation), suggesting that the primary defect might be in a radiosensitive prethymic precursor clone that needs the thymus to be expressed. The important role of the *lpr* gene in T cell proliferation, and consequently in the early SLE syndrome, has also been shown in genetic studies (1, 3, 16, 19). Because *lpr* is a recessive gene, its effects cannot be expressed in F_1 hybrids. However, when the *lpr* gene is established in a homozygous state among strains with late-developing disease, then early SLE develops; in +/+ mice, 50% mortality decreases from 17 to 5 mo of age, and in NZB mice the drop is from 16 mo to 5 mo (E. D. Murphy and J. D. Roths, personal communication). Using a different approach, Cowdery and Steinberg (20) found that crosses of CBA/N × MRL/1 F_1 males that express the *xid* gene had significantly lower levels of serum IgM than those of female littermates, but the presence of *xid* gene did not suppress spontaneously produced antibody to DNA. In further experiments with backcross males, these investigators demonstrated a direct association between lymphoproliferation (*lpr* gene) and spontaneous production of large amounts of anti-DNA. The latter results with *lpr/lpr* mice contrasted with those obtained by the same investigators (20, 21) and others (22) when testing NZB backcross males bearing the *xid* gene and producing very little IgM or anti-DNA antibody.

In contrast to the beneficial effects of thymectomy in *lpr/lpr* mice, our unpublished studies and those of others indicate that neonatal thymectomy accelerated the disease of NZB/W mice, especially males (17, 23–25). Moreover, athymic nude NZB/W mice developed disease at the same pace and as severely as their nu/+ controls (26). All these findings suggest an intrinsic B cell defect or polyclonal B cell activators as the responsible agent of disease in NZ mice and questioned the primary importance of the reported hormonal (5) and cellular (7, 27) defects of the NZ thymus in the development of SLE in this strain of mice. These results highlight once again the complex pathogenesis of murine SLE and reinforce our earlier proposal (28) that the cause and course of this disease in the various strains may rest on individual basic defects, different pathways of abnormalities, and distinct accelerating factors. Such an acceleratong factor acting on a background of autoimmune disease that develops late in life may modify it to an early-life one (3, 16, 19). The accelerating factors have now been defined as the *lpr* gene in MRL/Mp-*lpr/lpr* mice (1, 16–18; this study, and studies currently in progress), the Y chromosome-linked defect in BXSB male mice (1, 3, 16, 19, 29, 30), and possibly hormonal influences in NZB/W mice (31). As shown here, thymectomy blocked expression of the *lpr* accelerating factor and modified this disease of *lpr/lpr* substrain to a late disease or to no disease at all.

This study and studies underway strongly suggest that early, severe SLE-like disease in *lpr/lpr* mice is caused by abnormal stem cells, which upon differentiation in a thymus (irrespective of its genotype) give rise to proliferating Thy-1.2^+, Ly-1^+ cells (12, 32) that accelerate or help the expression of hypergammaglobulinemia, autoantibody production, and disease. This conclusion is consistent with results of previous studies suggesting that the proliferating cells in *lpr/lpr* mice exert an excessive helper function in vitro for polyclonal Ig synthesis (14) and anti-DNA antibody (33) production. Sequential studies remain to be performed so as to determine the exact onset of B cell hyperactivity in this substrain and its relationship to the expression of the *lpr* gene. In other words, it remains still to be ascertained whether expression of

the *lpr* gene has simply an accelerating effect on a preexisting B cell defect or whether it represents the actual triggering signal for expression of B cell activation and autoantibody production. At any rate, this is certainly the first animal model in which a single genetic defect has such a profound effect on the development of generalized autoimmunity. Congenic strains currently developed with single-gene defects provide a superb framework for comparison with normal genes at the same locus, for studying their interrelationship with known genes that control immunoregulation, and for linking genetic and cellular abnormalities as a whole with autoimmunity.

Summary

In young adulthood, MRL/Mp-*lpr/lpr* mice develop a severe systemic lupus erythematosus (SLE)-like syndrome associated with massive T cell proliferation. The congenic MRL/Mp-+/+ mice lack the *lpr* gene and develop chronic SLE late in life. We have exchanged thymic transplants between these substrains so as to determine the role of the thymus in the development of early, severe SLE and of lymphoproliferation. The median survival times of unmanipulated *lpr/lpr* and +/+ mice were 160 and 510 d, respectively. The *lpr/lpr* and +/+ mice thymectomized when newborn and transplanted at 1 mo with the opposite type of thymus retained the diseases phenotype of their unmanipulated counterparts with 50% mortality at 186 and 498 d, respectively. In contrast, *lpr/lpr* mice thymectomized when newborn but not transplanted with thymus did not develop lymphoid hyperplasia and glomerulonephritis, and 100% of them were alive at 390 d. Serologically, the thymectomized but untransplanted *lpr/lpr* mice had significantly reduced levels of autoantibodies, whereas thymectomized and transplanted mice of either substrain were similar to unmanipulated controls. The results indicate that: (*a*) a thymus is essential for expression of lymphoproliferation and early SLE-like disease in the *lpr/lpr* phenotype; (*b*) the *lpr/lpr* disease is not a result of a unique hormonal or microenvironmental defect(s) of the thymus of this substrain because the genotype of the thymus is irrelevant for the development of T cell proliferation and early SLE; (*c*) differentiation of +/+ stem cells under the hormonal or microenvrionmental influences of a thymus that possesses the *lpr* genotype does not lead to abnormal T cell differentiation or early autoimmunity; and (*d*) the *lpr/lpr* disease cannot be caused exclusively by an intrinsic B cell defect or environmental stimuli that cause B cell polyclonal activation.

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