Effects of Altered Prenatal Hormonal Environment on Expression of Autoimmune Disease in NZB/NZW Mice

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F_1 hybrid New Zealand Black (NZB) × New Zealand White (NZW) (NZB/NZW) mice spontaneously develop an autoimmune disease analogous to systemic lupus erythematosus (SLE). Testosterone exerts a powerful suppressive effect on this disorder in adult NZB/NZW mice. A series of experiments was designed to determine if disease would also be suppressed by exposing fetal NZB/NZW mice to increased testosterone. A model was developed in which NZB dams carrying NZB/NZW fetuses were treated with testosterone in a dose adequate to masculinize the external genitalia in female fetuses. NZB/NZW mice that were derived from testosterone-treated dams and control NZB/NZW offspring were followed in a longevity study and had serial assays to assess development of SLE. Additional experiments were carried out to measure lymphocyte subsets and responses to mitogens. Results were compared with F_1 hybrid offspring of C57BL/6 dams crossed with DBA/2 males, which are not autoimmune and do not develop SLE. Spleen cells from these groups were tested for Thy 1.2, CD4, CD8, and IgM receptors, and for responses to the mitogens Concanavalin A (ConA) and lipopolysaccharide. Control male NZB/NZW fetuses had unexpectedly high serum estradiol, which decreased significantly with maternal testosterone treatment. The testosterone-exposed male NZB/NZW fetuses developed into adults that lived longer than male NZB/NZW controls. Testosterone treatment of the dam was associated with elevated terminal anti-DNA levels but did not alter markers of renal disease in adult NZB/NZW mice of either sex. Testosterone-exposed NZB/NZW females had altered T-lymphocyte subsets and testosterone-exposed males had increased response to ConA compared to controls. In male NZB/NZW fetuses whose mothers were administered testosterone, the naturally high level of circulating estradiol observed in untreated male fetuses was decreased significantly. This decrease was associated with an increase in longevity. This unique observation has important implications for fetal exposure to endocrine disruptors in the environment. — Environ Health Perspect 104(Suppl 4):815–821 (1996)

Key words: autoimmunity, NZB mouse, C57BL/6 mouse, fetus, testosterone, estradiol, longevity

Introduction

Systemic lupus erythematosus (SLE) is a chronic and incurable disease characterized by formation of autoantibodies and immune-mediated damage to key target organs such as the kidney (1). The predilection of SLE for women of reproductive age (1), association between disease flares and pregnancy (2), aberrant pattern of estrogen metabolism (3), and relatively low levels of circulating testosterone in active disease (4) point to an important role of reproductive hormones in modulating SLE.

Matings between New Zealand Black (NZB) and New Zealand White (NZW) mice produce F_1 NZB/NZW hybrids, which develop a disease analogous to SLE. These animals produce antibodies directed against double-stranded DNA (anti-DNA) and die prematurely with glomerulonephritis and renal failure (5). Sex hormones are important modifying factors in the disease of NZB/NZW mice. Female NZB/NZW mice have early-onset disease and accelerated mortality, with mean longevity of 40 weeks (6), and autoantibody formation is accelerated by treatment with exogenous 17β-estradiol (7).

In contrast, androgens are beneficial in NZB/NZW mice. Males are expected to live longer than the females. The mean age of death in male NZB/NZW mice is 64 weeks (6), but early death occurs if male mice are castrated before puberty (7). Treatment with exogenous testosterone improves survival in male castrates (7). Testosterone therapy also improves survival in NZB/NZW females that are treated before or after the onset of renal disease, and ovariectomy is not necessary for female mice to receive therapeutic benefit (8,9).

Although testosterone modulates the severity of autoimmune disease in NZB/NZW mice, the critical periods at which key interactions occur between endogenous androgens and the immune system have not been completely defined. It seems logical to assume that the protective effects of naturally secreted androgens are exerted in the postpubertal period in males, when serum testosterone is found in high concentrations. Experimental evidence has shown, however, that early exposure to testosterone also has an important role in modifying the course of SLE in NZB/NZW males. Castration leads to acceleration of disease in male NZB/NZW mice if the surgery is performed at 2 weeks of age, but postpubertal castration is relatively ineffective (8,10). A recent report from this laboratory (11) showed that NZB/NZW males had little androgen receptor-mediated protection from severe disease between the ages of 6 weeks and 1 year.

Colborn et al. (12) have reviewed evidence that maternal exposure to endocrine-disrupting chemicals in the environment can permanently alter the functioning of developing systems, such as the immune system, in offspring due to transport of

chemicals from the mother during fetal life and lactation. A number of endocrine-disrupting chemicals typically encountered in water, air, and food and commonly found in human tissue are capable of permanently disrupting developing systems by binding to receptors for estrogen and androgen, which regulate the differentiation of many tissues; endocrine disruptors can either mimic or antagonize the actions of natural sex steroids (13–15). Thus, findings concerning the role of estrogen and androgen in the etiology of autoimmune disease are relevant to our understanding of the potential impact that exposure to endocrine disruptors during development can have on this disease.

This paper is the third in a series of publications that report the effects of hormonal manipulations on the developing immune system of NZB/NZW mice during prenatal life (16,17). A model was created in which NZB dams, pregnant with NZB/NZW fetuses, were treated in late pregnancy with testosterone or with the androgen blocker, flutamide. Both maternal treatments failed to produce long-lasting influences on female offspring, but the treatments were associated with increased longevity in the NZB/NZW males (16).

This paper focuses on gestational testosterone therapy in NZB dams and the influences of this treatment on their NZB/NZW offspring. Although maternal exposure to exogenous testosterone did not affect the lifespan in the NZB/NZW females produced by the treated dams, maternal testosterone treatment was associated with an unusual effect in the males. Unexpectedly, NZB/NZW males from androgen-implanted mothers had prolonged survival and lived significantly longer than comparable control males from untreated NZB dams (Figure 1) (16).

The hormonal status of the male NZB/NZW fetuses was unique. At the gestational age of 18 days, control males had very high serum estradiol that exceeded concentrations in littermate females by a factor of 2.6. Maternal treatment with testosterone significantly reduced (by one-third) serum estradiol concentrations in the male fetuses (Table 1) (17). Testosterone-treated NZB dams had significantly elevated serum testosterone (data not shown), but maternal treatment did not alter fetal serum testosterone levels (Table 1) (17).

We now describe serial assessments of disease progression, lymphocyte subsets, and responses to T-lymphocyte and B-lymphocyte mitogens in adult
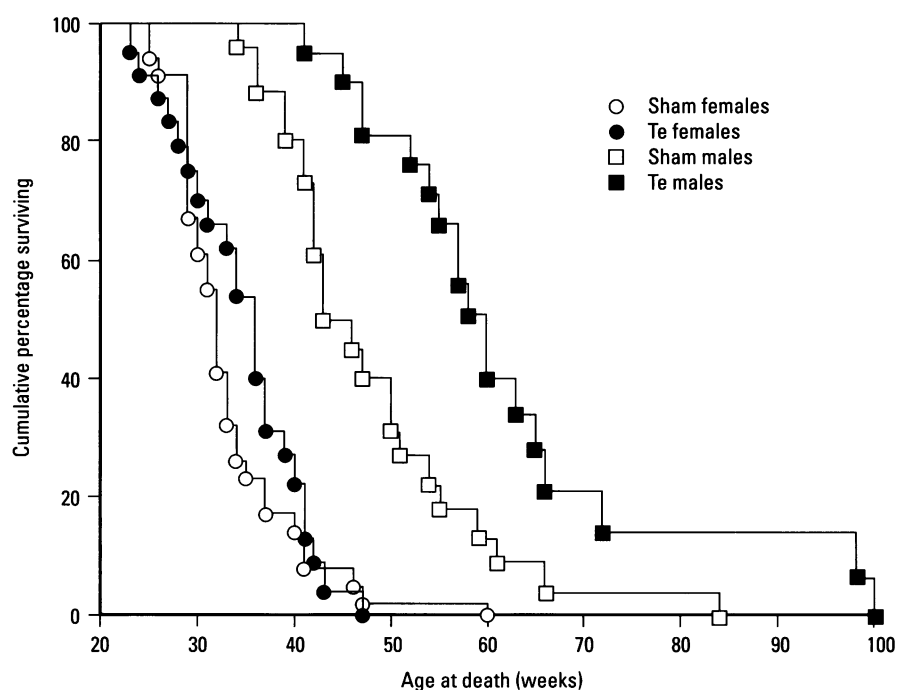
Figure 1. Survival in female and male NZB/NZW mice. Female mice and male mice were delivered by cesarean section from NZB dams that had been treated from days 13–18 of pregnancy with implanted testosterone, 0.75 mg. Control NZB/NZW females and males were delivered by cesarean section from sham-treated NZB dams. The mean age at death in female offspring of testosterone-treated dams did not differ significantly from offspring of control dams. Mean longevity in males from the dams treated with testosterone was prolonged (61 weeks±4) compared to males from control dams (48±2); p<0.001. Figure 1 is adapted from Keisler et al. (16) and is used with permission of the publisher.

Table 1. Serum estradiol and testosterone concentrations in NZB/NZW fetuses.[a]

Sex of offspring	Maternal treatment	Estradiol (pg/ml)	Testosterone (pg/ml)
Female	Testosterone	137 ± 44	2988 ± 408
	Sham	125 ± 44	1585 ± 762
Male	Testosterone	205* ± 42	1958 ± 485
	Sham	327 ± 42	2706 ± 829

[a]Offspring were delivered by cesarean section from NZB dams that received either subcutaneous implants containing testosterone or sham treatment. Values are expressed as mean ± 1 SEM. *$p < 0.05$ for male fetuses from testosterone-treated dams versus male fetuses from sham-treated dams. Data modified from Keisler et al. (17) and used with permission of the publisher.

NZB/NZW offspring of hormonally manipulated dams.

Materials and Methods

Animals

Autoimmune (SLE) Mice. New Zealand Black (NZB) females and New Zealand White (NZW) males (Jackson Laboratories, Bar Harbor, ME) were paired to produce NZB/NZW offspring.

Nonautoimmune Mice. C57BL/6 females and DBA/2 males (Harlan Sprague Dawley, Indianapolis, IN) were paired to produce C57BL × DBA/2 (C57/DBA2)

offspring. Animals were purchased at 5 weeks of age and maintained with an automatic 12-hr on, 12-hr off light cycle in the Research Service of the Harry S. Truman Memorial Veterans' Hospital under conditions described in an earlier publication (17). At 6 weeks of age, NZB females were paired with NZW males, and C57BL/6 females were paired with DBA/2 males. Females were checked daily for vaginal plugs, and the appearance of a plug was day 0.

Testosterone Treatment

Implants were made of 1-cm lengths (between the plugged ends) of Silastic tubing

(0.06-inch inner diameter, 0.125-inch outer diameter; Dow Corning, Midland, MI) filled with 0.75 mg testosterone (Sigma Chemical Co., St. Louis, MO) in 0.02 ml sesame oil. Fifteen NZB dams and eight C57BL/6 dams were anesthetized with Metofane (methoxyflurane) (Pitman-Moore, Inc., Mundelein, IL) and implanted on day 13 of pregnancy (16). The 0.75-mg dose of testosterone was chosen because it was determined in preliminary experiments that this was the lowest dose that significantly masculinized the external genitalia of female offspring. Masculinization was defined on the basis of increased anogenital space (the length between the anus and the genital papilla that becomes the scrotum in males). The dose was, however, low enough so that defeminization of female fetuses did not occur (i.e., these animals retained the capacity to ovulate) (16).

The vagina of the female mouse is usually open by 4 weeks of age, but it was anticipated that opening would be altered by prenatal exposure to exogenous testosterone (18). Vaginal opening did not occur in female F_1 offspring of testosterone-treated NZB and C57BL/6 dams by 12 weeks of age, and all of these offspring had hysterectomies (16) to prevent endometritis that would have occurred because of lack of uterine drainage. Ovaries were carefully left intact. This surgery did not cause expression of disease to differ, and data from these mice were pooled with data from other female NZB/NZW offspring of sham-treated dams (16).

Sham Treatment

Twelve NZB dams and 5 C57BL/6 dams received implants containing 0.02 ml sesame oil on day 13 of gestation, and 8 NZB dams and 4 C57BL/6 dams received injections of sesame oil–alcohol vehicle on days 13, 14, 15, 16, 17, and 18 of gestation. Offspring of dams that received either sham treatment had similar anogenital spaces and comparable expression of disease, and the results were combined.

Derivation of Offspring

Pups were delivered from all dams by cesarean section on day 18 of gestation, fostered to post-partum CF-1 mothers from the outbred colony of F. vom Saal, and weaned 21 days after birth (16).

Longevity Study of NZB/NZW Offspring

A longevity study was performed to determine if NZB dams exposed to testosterone

in the last third of pregnancy produced NZB/NZW offspring with altered expression of autoimmune disease. The following groups of NZB/NZW offspring were studied: female (n = 24) and male (n = 22) offspring of testosterone-treated dams and control female (n = 34) and male (n = 29) offspring of sham-treated dams.

Assessment of Active Autoimmune Disease

Mice were bled from the orbital plexus and urine was collected at 12, 24, 36, and 48 weeks of age. Mice were examined daily for signs of disease. They were bled, checked for albuminuria, sacrificed, and necropsied according to the protocol described in earlier publications when they developed neoplasms or appeared moribund (19–21).

Binding of heat-inactivated mouse serum to ^{14}C-labeled DNA derived from *Escherichia coli* (Amersham Corporation, Arlington Heights, IL) was measured in a modified Farr assay. Values greater than 20% binding indicated the presence of anti-DNA (22,23). Urine was tested for albuminuria with Albustix (Ames Co., Elkhart, IN) and graded on a scale of 0 to 4+ according to five colors on a chart provided by the manufacturer. Results were classified as 0, 1+ (30 mg/dl), 2+ (100 mg/dl), 3+ (300 mg/dl), or 4+ (>2000 mg/dl). Albuminuria greater than 2+ was considered significant. Blood urea nitrogen (BUN) was assayed using a colorimetric assay kit (American Monitor Corp., Indianapolis, IN). In this laboratory, the mean BUN level in stored frozen sera was 30 mg/dl ± 4 SEM for normal female mice and 35 mg/dl ± for normal male mice (24).

Lymphocyte Enumeration and Mitogenic Responses

Groups of mice, each group containing 5 NZB/NZW hybrids and 5 C57/DBA2 hybrids of each sex from testosterone-treated or control dams, were studied at 8 weeks and 16 weeks of age. These animals were not included in the longevity study.

Monoclonal Antibodies and Antisera

Biotin-conjugated anti-Thy 1.2, phycoerythrin (PE)-conjugated anti-L3T4 (CD4), fluorescein isothiocyanate (FITC)-conjugated anti-Lyt 2 (CD8), and PE-conjugated streptavidin were purchased from Becton Dickinson (Mountain View, CA). Other reagents were FITC-conjugated affinity purified F(ab')2 sheep anti-mouse IgG (Cooper Biomedical, Inc., Malvern, PA), and biotinylated F(ab')2 fragment rabbit

anti-mouse IgM (Zymed Laboratories, South San Francisco, CA).

Preparation of Cells

Single cell suspensions were prepared by mechanically dissociating spleens in RPMI 1640 (Gibco, Grand Island, NY). The cells were filtered through nylon mesh and washed with RPMI 1640 enriched with 0.5% fetal calf serum (FCS) (Gibco), L-glutamine, penicillin, and streptomycin. Lymphocytes were isolated on a density gradient (Ficoll-Paque, Pharmacia, Piscataway, NJ). Cells were diluted to 1×10^6/ml, and 0.1-ml aliquots were added to 12×75-mm plastic test tubes and washed in phosphate-buffered saline (PBS). Cells were stained with monoclonal antibodies at saturating concentrations (0.2–5 µg) at 4°C for 30 min. PE-streptavidin was added to tubes containing biotinylated antibodies. After staining was complete, cells were washed twice with PBS containing FCS, washed a third time with PBS without FCS, fixed in 0.1 ml 0.5% paraformaldehyde, and examined 16 hr later by flow cytometric analysis.

A FACScan flow cytometer (Becton Dickinson) was used under standardized conditions for measurement of light scatter and fluorescence (25). Fluorescence signals were collected at 515 ± 10 nM (FITC) and 600 ± 15 nM (PE) in single- or dual-parameter histogram format from 10,000 cells with the lymphocyte light scatter gates, as defined by forward and perpendicular light scatter signals. Data were expressed as the percentage of cells bearing the antigen of interest. When necessary, nonspecific staining was subtracted from positive cells.

Lymphocyte Transformation

Mitogenic responses of splenocytes to Concanavalin A (ConA) (Pharmacia), 0.5 µg, or lipopolysaccharide (LPS) (*E. coli* 026:B6; Difco, Detroit, MI), 40 µg, were assayed by incubating the cells with either ConA or LPS to stimulate cell division, pulsing the dividing cells with ^{3}H-thymidine, harvesting the cells on glass fiber filter paper, and using recovered CPM as a measure of cell proliferation. This assay was performed as described in an earlier publication (24), except that phenol red-free RPMI 1640 media (Gibco, Grand Island, NY) was used. Mean values of triplicate samples were determined, and results were expressed as differences in counts per minute (ΔCPM) between stimulated and unstimulated cultures.

Statistical Analyses

Analysis of variance procedures and mean comparison tests were used to determine differences in dependent variables in each experiment (General Linear Models, SAS Institutes, Cary, NC). Planned comparisons were made using least significant difference procedures (26).

Results

Longevity Study of NZB/NZW Offspring

Testosterone treatment of NZB dams in the last third of gestation affected longevity of the offspring in a sex-dependent manner. Figure 1 illustrates cumulative survival in NZB/NZW females and males that were delivered from testosterone-treated or sham-treated control dams. The major cause of death in mice of both sexes was SLE, manifested as renal disease leading to renal failure and vasculitis (16). Age at death in female offspring of testosterone-treated dams was 34 ± 1 week; longevity in this group did not differ from female offspring of control dams (37 ± 2 weeks). In contrast, outcome in NZB/NZW males was affected by the maternal testosterone therapy. Males from testosterone-treated dams had lifespans of 61 ± 4 weeks versus male offspring of control dams (48 ± 2 weeks; $p < 0.001$) (16).

Assessment of Active Autoimmune Disease

Serial assays of anti-DNA, an important marker of disease activity in NZB/NZW mice (27), are shown in Table 2. All female offspring had the expected age-dependent increase in anti-DNA concentration. In females produced by the testosterone-treated NZB dams, anti-DNA appeared to exceed control values at 36 weeks of age; this increase was significant in serum that was collected at spontaneous death. Anti-DNA concentrations in male offspring also increased with age and were not altered by testosterone treatment of the dam.

Progression of kidney disease was not changed by perinatal exposure to testosterone in mice of either sex. Table 3 illustrates albuminuria, which appeared in female and male mice by 24 weeks of age. Albuminuria was not accelerated in offspring of testosterone-treated dams. Terminal increases in BUN concentrations reflected the development of renal failure at the time of spontaneous death in NZB/NZW mice of both sexes (Table 3).

Table 2. Anti-DNA in NZB/NZW mice.[a]

		Anti-DNA (% binding)				
		Age (weeks)				
Sex of offspring	Maternal treatment	12	24	36	48	Terminal
Females	Testosterone	8 ± 1	11 ± 2	61 ± 7	ND	60 ± 6*
	Sham	10 ± 2	11 ± 2	49 ± 10	ND	45 ± 5
Male	Testosterone	9 ± 1	10 ± 2	28 ± 5	45 ± 5	42 ± 9
	Sham	12 ± 1	9 ± 2	30 ± 5	46 ± 8	26 ± 6

ND, not determined. [a]Antibodies to ds-DNA are expressed as percent ^{14}C-DNA bound to mouse serum. Values greater than 20% are abnormal. Results are expressed as mean $\pm$ 1 SEM. *$p < 0.05$ for DNA binding in terminal serum from female offspring of testosterone-treated dams versus female offspring of sham-treated dams.

Table 3. Parameters of renal disease in NZB/NZW mice.[a]

		Albuminuria				
		Age (weeks)				
Sex of offspring	Maternal treatment	12	24	36	48	Terminal
Female	Testosterone	0	23	70	ND[a]	76
	Sham	0	17	40	ND	88
Male	Testosterone	0	4	0	35	56
	Sham	0	0	20	43	65
		BUN				
Female	Testosterone	23 ± 1	29 ± 2	36 ± 4	ND	154 ± 17
	Sham	22 ± 1	26 ± 2	27 ± 6	ND	138 ± 14
Male	Testosterone	23 ± 2	22 ± 2	20 ± 3	30 ± 7	127 ± 13
	Sham	27 ± 1	22 ± 2	30 ± 3	47 ± 10	83 ± 16

ND, not determined. [a]Albuminuria is shown as percent of surviving mice with 3+ to 4+ readings on Albustix. BUN results are milligram per decaliter and expressed as mean $\pm$ 1 SEM. Only one female mouse was alive at the age of 48 weeks.

Lymphocyte Populations in NZB/NZW Mice

Table 4 displays percentages of cells with surface markers for T lymphocytes (Thy 1.2, CD4, and CD8) and B lymphocytes (IgM). At 8 weeks of age, female NZB/NZW mice from testosterone-treated dams had a lower percentage of splenocytes expressing Thy 1.2 compared to NZB/NZW females from sham-treated dams (18 vs 24%; $p < 0.01$). Treating NZB dams with testosterone was associated with similar reduction in the proportion of CD4+ cells in female offspring ($p < 0.05$). No significant differences were found between percentages of cells bearing CD8 or IgM in offspring of dams that received testosterone or sham treatment. Data from 16-week-old mice are not shown; at this point, NZB/NZW offspring from treated or sham-treated dams did not differ with respect to T-lymphocyte or B-lymphocyte markers.

Lymphocyte Populations in C57/DBA2 Mice

Percentages of spleen cells from 8-week-old C57/DBA2 mice that reacted with the four antibodies of interest are in Table 4. Testosterone treatment of C57BL/6 dams did not appear to affect proportions of cells bearing each marker. Likewise, offspring from treated and control dams, studied at 16 weeks of age, did not differ with respect to cell surface antigens (data not shown).

Mitogenic Responses in NZB/NZW Mice

Proliferation of NZB/NZW spleen cells in response to the T-lymphocyte mitogen, ConA, and the B-lymphocyte mitogen, LPS, are shown in Table 5. At 8 weeks of age, there was a trend for increased ^{3}H-thymidine uptake in response to both mitogens in female and male offspring of testosterone-treated dams. This increase was significant only for ConA-cultured spleen cells from the testosterone-exposed NZB/NZW males, compared to male offspring of sham-treated dams ($p < 0.05$). ConA and LPS produced equivalent responses in NZB/NZW mice of both sexes, at 8 and 16 weeks of age.

Table 4. Cell surface antigens on spleen cells from 8-week-old NZB/NZW and C57/DBA2 mice.[a]

F_1 Hybrid	Sex of offspring	Maternal treatment	% Positive cells			
			Thy 1.2	CD4	CD8	IgM
NZB/NZW	Female	Testosterone	18 ± 2*	16 ± 1**	7 ± 0.5	60 ± 3
		Sham	24 ± 2	18 ± 1	7 ± 0.5	61 ± 3
	Male	Testosterone	23 ± 2	17 ± 1	6 ± 0.5	58 ± 3
		Sham	22 ± 2	16 ± 1	7 ± 0.6	50 ± 4
C57/DBA2	Female	Testosterone	18 ± 5	13 ± 1	6 ± 1	70 ± 3
		Sham	17 ± 5	13 ± 1	6 ± 1	70 ± 3
	Male	Testosterone	16 ± 5	13* ± 1	6 ± 1	69 ± 3
		Sham	27 ± 5	12 ± 1	5 ± 1	68 ± 3

[a]Percentages of spleen cells with positive staining for each antigen are expressed as mean ± 1 SEM. *$p < 0.01$ and **$p < 0.05$ for female NZB/NZW offspring of testosterone-treated dams versus female NZB/NZW offspring of sham-treated dams.

Table 5. Responses to mitogenic stimulation with ConA and LPS in 8-week-old and 16-week-old NZB/NZW and C57/DBA2 mice[a]

F_1 Hybrid	Sex of offspring	Maternal treatment	8 weeks		16 weeks	
			ConA	LPS	ConA	LPS
NZB/NZW	Female	Testosterone	105 ± 7	45 ± 4	68 ± 4	31 ± 2
		Sham	89 ± 11	33 ± 6	66 ± 1	32 ± 3
	Male	Testosterone	97 ± 10*	41 ± 7	67 ± 4	36 ± 2
		Sham	77 ± 13	27 ± 6	68 ± 4	33 ± 3
C57/DBA2	Female	Testosterone	57 ± 19	30 ± 7	41 ± 18	30 ± 4
		Sham	59 ± 17	46 ± 3	53 ± 15	36 ± 8
	Male	Testosterone	35 ± 16	47 ± 3	54 ± 15	34 ± 6
		Sham	36 ± 16	43 ± 4	52 ± 16	37 ± 5

[a]CPM × 10^3. Incorporation of ^{3}H-thymidine by dividing cells is expressed as mean ± 1 SEM of Δ CPM (CPM in stimulated culture − CPM in unstimulated culture). *$p < 0.05$ for female offspring of testosterone-treated dams versus female offspring of sham-treated dams.

Mitogenic Responses in C57/DBA2 Mice

In nonautoimmune C57/DBA2 hybrids, active responses were observed when spleen cells were cultured with either mitogen. Response were not influenced by prenatal treatment, sex, or age (Table 5).

Discussion

Gonadal hormones mediate severity of autoimmune disease in NZB/NZW mice, but the mechanisms and timing of the most important interactions with the immune system have not previously been defined. Estrogens are capable of suppressing natural killer cell cytotoxic activity (28) and stimulating intense B-cell activity (28,29) in NZB/NZW mice. Androgenic hormones exert widespread influences on cell-mediated immunity, sustaining interleukin (IL)-2 production (30) and increasing T-cell activity in castrated males (31).

It may be reasoned that hormones exert modulating effects within the developing immune system of the fetus, whereas other important influences occur when concentrations of hormones change at puberty or after the animal has reached sexual maturity. We have investigated hormonal interactions in the last third of gestation, a period in which gonadal steroids regulate the differentiation of numerous organ systems.

Several unique models have been developed in this laboratory to facilitate studies of altered prenatal hormone concentrations on the subsequent course of autoimmune disease in NZB/NZW mice. In the testosterone implant model, pregnant NZB dams are treated on days 13 to 18 of gestation with implanted testosterone in a dose that somewhat masculinizes genitalia of the female NZB/NZW offspring (16). This treatment results in long-lived male NZB/NZW fetuses that survive for a significantly longer period compared to NZB/NZW males from control dams (16).

This present paper presents measurements of three parameters of active autoimmune disease in female and male NZB/NZW offspring of testosterone-treated and control dams. Anti-DNA, albumin-

uria, and BUN were assayed serially during the first year of life. These indicators increased as expected as the animals grew older. At 36 weeks of age, female NZB/NZW mice from testosterone-treated dams had active production of anti-DNA (mean = 61%). Anti-DNA remained elevated in the terminal phase of SLE in this group (60% vs 45% in females from sham-treated dams; $p < 0.05$). The increased anti-DNA concentration, a marker of active disease, was not associated with accelerated mortality in the females from hormone-treated dams.

This analysis suggested that prenatal exposure to exogenous androgens was associated with accelerated autoantibody formation in the females. Paradoxically, their lifespans were not shortened compared to same-sex controls. In the corresponding male littermates, testosterone-exposed offspring did survive longer than expected in the face of active autoimmune disease and renal insufficiency. Their increased longevity was compatible with the existence of a protective factor or factors that prevented early mortality in the face of active anti-DNA formation. Death was delayed in these males even though they had long-standing, active disease that paralleled males from sham-treated dams.

Our understanding of the protective effects that the prenatal hormone milieu may exert on longevity is confounded by changes that occur as the mice become adults. Females produce cyclic estrogen and prolactin, which are immunostimulatory, and males produce immune-suppressing androgens. We are currently examining mechanisms by which the prenatal hormonal environment could interact with post-pubertal hormone surges in adult NZB/NZW mice to prolong survival in animals with active SLE.

Additional groups of NZB/NZW offspring of testosterone-treated and control dams were tested for percentages of cells bearing T-lymphocyte or B-lymphocyte markers and for mitogenic responses to ConA and LPS. Results were compared with a genetically distinct F_1 hybrid (C57/DBA2) that does not develop autoimmune disease. Of interest, 8-week-old female NZB/NZW offspring of testosterone-treated dams had significant reductions in percentages of spleen cells bearing surface antigens that are characteristic of T lymphocytes (Thy 1.2), and the helper/inducer T-lymphocyte subset (CD4). This apparent reduction in T lymphocytes, however, did not suppress responses to the

T-lymphocyte mitogen, Con-A. The difference in T-lymphocyte subsets did not persist to 16 weeks of age, and was not noted in NZB/NZW males or C57/DBA2 hybrids. Male NZB/NZW mice from treated dams had no changes in lymphocyte subsets, but a significant increase in response to ConA was noted at 8 weeks of age.

Maternal testosterone treatment did have some longstanding effects on lymphoid cell subsets and responses to mitogens. In testosterone-exposed female NZB/NZW offspring, the reduced numbers of CD4+ T-helper cells at 8 weeks of age may have represented either a lag in production, or increased destruction of a key group of lymphocytes that is needed to sustain autoimmune disease in NZB/NZW mice (32). The relative paucity of CD4+ cells could have been protective, in that it might have affected expression of disease and resulted in some prolongation of life in this group of female mice with very high anti-DNA levels. On the other hand, the trend to increased responses to ConA and LPS in females and the significant increase in ConA responsiveness in males from testosterone-treated dams argues for a transient increase in activity of the immune system at an early age. The significance of this response is not clear; it could reflect an altered production of lymphocyte growth factors that may or may not be associated with the phenomenon of increased longevity in testosterone-exposed NZB/NZW males.

Our evaluations of the immune system in these animals did not identify a single protective factor that could have extended longevity in testosterone-exposed NZB/NZW mice. Future investigations will focus on identifying changes in the prenatal and postnatal hormonal environments that may have affected the genesis of lymphocyte subsets or reset these cells to produce immune mediators that have protective as well as immune-stimulating properties. Additional studies are required to define lymphocyte profiles and function in juvenile animals, and to separate the effects of prenatal hormone exposure from immunosuppressive actions of endogenous androgens.

In the course of investigating the hormonal environment in which fetal NZB/NZW mice develop, we discovered that unmanipulated NZB/NZW males had unprecedented elevation of serum estradiol on day 18 of fetal life. For comparison, the observed concentration of serum estradiol in 18-day C57/DBA2 fetuses was (mean ± SEM) 158 pg/ml ± 48 for females and 183 pg/ml ± 38 for males. Values for CF-1 mouse fetuses at 18 days of gestation were females, 113 pg/ml ± 7 and males, 90 pg/ml ± 4 (33). In the male NZB/NZW fetuses, the concentration of estradiol, (mean ± SEM) 327 pg/ml ± 42, greatly exceeded that of the female NZB/NZW littermates (125 pg/ml ± 44), $p < 0.01$. Testosterone treatment of the NZB dams decreased serum estradiol in male NZB/NZW fetuses to (mean) 205 pg/ml (17).

The maternal treatment that decreased fetal estradiol in the NZB/NZW males did not alter their circulating testosterone, but resulted in significant prolongation of lifespans. These findings lead to the prediction that increased concentrations of estradiol during fetal life are related to early mortality from active autoimmune disease in NZB/NZW males.

The findings that NZB/NZW male fetuses have elevated serum estradiol compared to concentrations in male fetuses in nonautoimmune strains (16,17,34) and that a decrease in serum estradiol during fetal life in NZB/NZW males was associated with an increase in longevity, have important implications for studies of environmental influences on autoimmune disease. Our findings suggest that environmental endocrine disrupting chemicals that can bind to estrogen receptors and mimic the action of estradiol may affect the fetus and alter the subsequent course of autoimmune disease.

Numerous chemicals present in food, water, and air can bind to estrogen receptors and are referred to as environmental estrogens. Man-made environmental chemicals with estrogenic activity include pesticides, such as DDT and methoxychlor, components of commonly used products, such as plastics and soaps, and the epoxy lining of cans, such as octylphenol, nonylphenol and bisphenol-A (14,15). Environmental estrogens synthesized by man differ from naturally occurring plant estrogens (phytoestrogens) in that only man-made chemicals persist while stored in fat for prolonged periods after ingestion; phytoestrogens are rapidly cleared from the circulation (14). Thus, maternal exposure to persistent environmental estrogens before or during pregnancy and lactation can result in exposure of offspring to the chemicals during differentiation of critical organ systems.

While it has been recognized for some time that endogenous steroids modulate immune function and are involved in sex differences in immune function, relatively little attention has been paid to the effects of exposure to steroids during critical periods in the organization of the developing immune system on subsequent immune function. Steroids exert organizational effects on differentiating tissues, including tissues in the immune system, such that the functioning of the tissue throughout the remainder of life is permanently altered as a result of a change in hormone levels during differentiation (35).

The possibility that exposure of fetuses or newborns to endocrine-disrupting chemicals might be involved in a host of diseases associated with disfunction in the immune system needs to be examined. Information concerning parental exposure to endocrine disruptors can then be analyzed in relation to diseases that do not become manifest until the offspring are adults.

REFERENCES

1. Lahita RG, ed. Systemic Lupus Erythematosus. 2d ed. New York:Churchill Livingstone, 1992.
2. Petri M, Howard D, Repke J. Frequency of lupus flare in pregnancy. The Hopkins Lupus Pregnancy Center experience. Arthritis Rheum 34:1538–1545 (1991).
3. Lahita RG, Bradlow L, Fishman J, Kunkel HG. Estrogen metabolism in systemic lupus erythematosus: patients and family members. Arthritis Rheum 25:843–846 (1982).
4. Lahita RG, Bradlow HL, Ginzler E, Pang S, New M. Low plasma androgens in women with systemic lupus erythematosus.

Arthritis Rheum 30:241–248 (1987).
5. Andrews BS, Eisenberg RA, Theofilopolous AN, Izui S, Wilson CB, McConahey PJ, Murphy ED, Roths JB, Dixon FJ. Spontaneous murine lupus like syndromes. Clinical and immunopathological manifestations in several strains. J Exp Med 148:1198–1215 (1978).
6. Howie JB, Helyer BJ. The immunology and pathology of NZB mice. Adv Immunol 9:215–266 (1968).
7. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival,

antinucleic acid antibodies, and glomerulonephritis in NZB/NZW F$_1$ mice. J Exp Med 147:1568–1583 (1978).

8. Melez KA, Boegel WA, Steinberg AD. Therapeutic studies in New Zealand mice. VII: Successful androgen treatment of NZB/NZW F$_1$ females of different ages. Arthritis Rheum 23:41–47 (1980).

9. Roubinian JR, Papoian R, Talal N. Androgenic hormones modulate autoantibody responses and improve survival in murine lupus. J Clin Invest 59:1066–1070 (1977).

10. Steinberg AD, Melez KA, Raveche ES, Reeves JP, Boegel WA, Smathers PA, Taurog JD, Weinlein L, Duvic M. Approach to the study of the role of sex hormones in autoimmunity. Arthritis Rheum 22:1170–1176 (1979).

11. Walker SE, Besch-Williford CL, Keisler DH. Accelerated deaths from systemic lupus erythematosus in NZB × NZW F$_1$ mice treated with the testosterone-blocking drug flutamide. J Lab Clin Med 124:401–407 (1994).

12. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384 (1993).

13. Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM. Persistent DDT metabolite p,p'DDE is a potent androgen receptor antagonist. Nature 375:581–585 (1995).

14. vom Saal FS. Environmental estrogenic chemicals: their impact on embryonic development. Hum Ecol Risk Assess 1:3–15 (1995).

15. vom Saal FS, Nagel SC, Palanza P, Boechler M, Parmigiani S, Welshons WV. Estrogenic pesticides: binding relative to estradiol in MCF-7 cells and effects of exposure during fetal life on subsequent territorial behaviour in male mice. Toxicol Lett 77:343–350 (1995).

16. Keisler LW, vom Saal FS, Keisler DH, Walker SE. Hormonal manipulation of the prenatal environment alters reproductive morphology and increases longevity in autoimmune NZB/W mice. Biol Reprod 44:707–716 (1991).

17. Keisler LW, vom Saal FS, Keisler DH, Rudeen PK, Walker SE. Aberrant hormone balance in fetal autoimmune NZB/W mice following prenatal exposure to testosterone excess or the androgen blocker, flutamide. Biol Reprod 53:1190–1197 (1995).

18. Bok G, Drews U. The role of the Wolffian ducts in the formation of the sinus vagina: an organ culture study. J Embryol Exp Morph 73:275–295 (1983).

19. Walker SE, Bole GG Jr. Augmented incidence of neoplasia in NZB/NZW mice treated with long-term cyclophosphamide. J Lab Clin Med 82:619–6 33 (1973).

20. Walker SE, Bole GG, Jr. Influence of natural and synthetic estrogens on the course of autoimmune disease in the NZB/NZW mouse. Arthritis Rheum 16:231–239 (1973).

21. Walker SE, Kier AB, Siegfried EC, Harris BG, Schultz JS.

Accelerated autoimmune disease and lymphoreticular neoplasms in F$_1$ hybrid PN/NZB and NZB/PN mice. Clin Immunol Immunopathol 39:81–92 (1986).

22. Walker SE, Bole GG, Jr. Selective suppression of autoantibody responses in NZB/NZW mice treated with long-term cyclophosphamide. Arthritis Rheum 18:265–272 (1975).

23. Walker SE, Bole GG, Jr. Suppressed heterogeneous antinuclear antibody response in lymphoma-bearing NZB/NZW mice. Clin Exp Immunol 24:210–217 (1976).

24. Walker SE, Hewett JE. Responses to T-cell and B-cell mitogens in autoimmune Palmerston North and NZB/NZW mice. Clin Immunol Immunopathol 30:469–478 (1984).

25. Caldwell CW, Maggi J, Henry LB, Taylor HM. Fluorescence intensity as a quality control parameter in clinical flow cytometry. Am J Clin Pathol 88:447–456 (1987).

26. Snedecor GW, Cochran WG. Statistical Methods. 8th ed. Ames:Iowa State University Press, 1989.

27. Steinberg AD, Pincus T, Talal N. DNA-binding assay for detection of anti-DNA antibodies in NZB/NZW F$_1$ mice. J Immunol 102:788–790 (1969).

28. Nilsson N, Carlsten H. Estrogen induces suppression of natural killer cell cytotoxicity and augmentation of polyclonal B cell activation. Cell Immunol 158:131–139 (1994).

29. Carlsten H, Tarkowski A. Histocompatibility complex gene products and exposure to oestrogen: two independent disease accelerating factors in murine lupus. Scand J Immunol 38:341–347 (1993).

30. Dauphinee MJ, Kipper S, Roskos K, Wofsy D, Talal N. Androgen treatment of autoimmune NZB/NZW mice enhances IL-2 production. Arthritis Rheum 24:S64 (1981).

31. Weinstein Y, Berkovich Z. Testosterone effect on bone marrow, thymus, and suppressor T cells in the (NZB × NZW)F$_1$ mice: Its relevance to autoimmunity. J Immunol 126:998–1002 (1980).

32. Connolly K, Roubinian JR, Wofsy D. Development of murine lupus in CD4-depleted NZB/NZW mice. Sustained inhibition of residual CD4$^+$ T cells is required to suppress autoimmunity. J Immunol 149:3083–3088 (1992).

33. Montano MM, Welshons WV, vom Saal FS. Free estradiol in serum and brain uptake of estradiol during fetal and neonatal sexual differentiation in female rats. Biol Reprod 53:1198–1207 (1995).

34. vom Saal FS. Sexual differentiation in litter-bearing mammals: influence of sex of adjacent fetuses in utero. J Anim Sci 67:1824–1840 (1989).

35. vom Saal FS, Montano MM, Wang MH. Sexual differentiation in mammals. In: Chemically-induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Princeton, NJ:Princeton Scientific Publishing, 1992;17–83.