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

Sara E. Walker,¹ Lydia W. Keisler,² C. William Caldwell,³ Ann B. Klor,⁴ and Frederick S. vom Saal¹

¹Department of Internal Medicine, University of Vermont, Colchester, Vermont; ²Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas; ³Department of Biological Sciences, University of Vermont, Burlington, Vermont; ⁴Department of pathology and Anatomical Sciences, University of Vermont, Burlington, Vermont; ⁵Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas.

Introduction

Systemic lupus erythematosus (SLE) is a chronic and incurable disease characterized by formation of autoantibodies and immune-mediated damage to target organs such as the kidney (1). The predilection of SLE for women of reproductive age (2), association between disease flares and pregnancy (2), diverse 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 SLE.

Mating between New Zealand Black (NZB) and New Zealand White (NZW) mice yields NZB/NZW hybrids, which develop a disease analogous to SLE. These animals produce antibodies directed against double-stranded DNA (anti-dsDNA) 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 accelerate mortality, with mean longevity of 49 weeks (6), and autoantibody formation is accelerated by treatment with agonist 17β-estradiol (7).

In contrast, androgens are beneficial in NZB/NZW mice. Male mice are expected to live longer than the females. The mean age of death in male NZB/NZW mice is 66 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 estradiol is not necessary for female mice to receive therapeutic benefit (8).

Although testosterone modulates the severity of autoimmune disease in NZB/NZW mice, the critical periods at which key interactions occur between endogenous estrogen and systemic hormones have not been completely defined. It seems logical to assume that the protective effects of testosterone may be enhanced in the postpartum period in males, when serum testosterone is found in high concentrations. Experimental evidence has shown, however, that early exposure to testosterone is ineffective in modifying the course of SLE in NZB/NZW mice. 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 (9).

A recent report from this laboratory (10) showed that NZB/NZW males had higher androgen receptor-mediated protection from severe disease between the ages of 6 weeks and 1 year.

1-Carpenter 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 town sludge are capable of per- manently 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 onset of autism spectrum disorder are relevant to our understand- ing of the potential impact that expo- sure to endocrine disruptors during develop- ment can have on this disease.

This paper is the third in a series of publications that report the effects of hor- monal manipulations on the developing immune system of NZB/NZW mice during perinatal life (16,17). A model was cre- ated in which NZB dams, pregnant with NZB/NZW fetuses, were treated in late pregnancy with testosterone or with the antiandrogen bicalutamide. Both mater- nal treatments failed to produce long-last- ing 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 testosterone in utero did not affect the lifespan in the NZB/NZW females produced by the treated dams, maternal testosterone treatment was associ- ated with an unusual effect in the males. Unexpectedly, NZB/NZW males from androgen-implanted mothers had pro- longed survival and lived significantly longer than the control males from untreated NZB dams (Figure 1) (16,17).

The hormonally treated males of the NZB/NZW NZB/NZW offspring was suspect. At the ger- 

Table 1. Serum androgen and testosterone concentrations in NZB/NZW littermates a

<table>
<thead>
<tr>
<th>Sex of offspring</th>
<th>Maternal treatment</th>
<th>Testosterone (nmol/L)</th>
<th>Androstenedione (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Testosterone</td>
<td>138 ± 44</td>
<td>3888 ± 42</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>123 ± 44</td>
<td>1985 ± 30</td>
</tr>
<tr>
<td>Male</td>
<td>Testosterone</td>
<td>205 ± 42</td>
<td>3188 ± 285</td>
</tr>
<tr>
<td></td>
<td>Androstenedione</td>
<td>205 ± 42</td>
<td>2760 ± 250</td>
</tr>
</tbody>
</table>

*Offspring were delivered by same-sex litters from NZB dams that received either testosterone implants con- taining testosterone or sham treatment. Values are expressed as mean ± S.E. (n = 10) for male litters from testosterone-treated dams versus male litters from sham-treated dams. Data adapted from Kasturi et al. (17) and used with permission of the publisher.

Figure 1. Survival in female and male NZB/NZW mice. Female mice and male mice were delivered by same-sex litters from NZB dams that had been treated with or without testosterone from days 13-18 of pregnancy, then studied to determine longevity from birth. Male longevity in litters from dams treated with testosterone was prolonged (p < 0.001) as compared to controls (dams killed at 8 months). All mice were at least 12 months of age at the time of study. Data adapted from Kasturi et al. (17) and used with permission of the publisher.

NZB/NZW offspring of hormonally manipulated dams.

Materials and Methods

Animals

Antimouse (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. Nanaemia (Langerhans) mice, C57BL/6 females and B10.D2 females (Harlan Sprague Dawley, Indianapolis, IN) were paired to produce C57BL × B10.D2 females (C57BL/D2) and C57BL/6 females and B10.D2 males (Harlan Sprague Dawley, Indianapolis, IN) were paired to produce C57BL × B10.D2 males (C57BL/D2).

offspring. Animals were purchased at 5 weeks of age and maintained with an auto- matic 12-hr on-12-hr off light cycle in the research Services of the Harry S. Truman Memorial Veterans' Hospital under condi- tions 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 B10.D2 males. Females were caged daily for vaginal plugs, and the appearance of a plug was day 0.

Testosterone Treatment

Implants were made of 1-cm lengths (between the clamped ends) of Silastic tubing
(0.06-inch inner diameter, 0.125-inch outer diameter, M1) filled with 0.75 mg testosterone (Sigma Chemical Co., St. Louis, MO) in 0.02 ml saline oil. Fifteen NZB dams and eight C57BL/6 dams were anesthetized with Ketamine (Ketaset) and Na pentobarbital (Workman-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 as the presence of increased anogenital space (the length between the anus and the genital papilla that becomes the clitoris in males). The dose was, however, low enough so that feminization of female fens 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 delayed by prenatal exposure to exogenous testosterone (18). Vaginal opening did not occur in female F1 offspring of testosterone-treated NZB and C57BL/6 dams by 12 weeks of age, and all of these offspring had hypogonadism (16) so no prenatal exposure that would have occurred because of lack of ovarian function. These fens were therefore castrated left intact. This surgery did not cause expression of disease to differ, and data from these mice were combined with data from other female NZB/NZW offspring of sham-operated dams (16).

Sham Treatment

Two control litters of C57BL/6 dams received implants containing 0.02 ml saline oil on day 13 of gestation, and 8 NZB dams and 6 C57BL/6 dams received injection of saline oil-alcohol vehicle on days 13, 14, 15, 16, 17, and 18 of gestation. The three mice of each dam were killed with ether 8 weeks after birth. No histopathologic differences were noted.

Derivation of Offspring

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

Longevity Study of NZB/NZW Mice

A longevity study was performed to deter- mine if NZB dams exposed to testosterone in the last third of pregnancy produced NZB/NZW offspring with altered expres- sion of autoimmune disease. The following groups of NZB/NZW offspring were stud- ies: female (n = 24) and male (n = 22) offspring of testosterone-treated dams and control female (n = 34) and male (n = 29) offspring of sham-operated 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. Nows were examined daily for signs of disease. They were bled, checked for albuminuria, and necropsied according to the protocol described in earlier publications when they developed neoplasms or appeared moribund (19-21).

Rinding of heat-inactivated mouse serum to 3H-label DNA derived from Ehrlichia carcini (American Type Culture Collection, Rockville, MD) was measured in a modified Farr assay. Volumes greater than 20% binding indicated the presence of anti- DNA (22,23). Urine was tested for albumi- inuria with Albu-stix (Ames Co., Elkhart, IN) and graded on a scale of 0 to 4 accord- ing to five colors on a chart provided by the manufacturer. Results were classified as 0, 1, 2, 3, and 4 (progressively greater than 2) was considered significant.

Liver urea nitrogen (BUN) was assayed using a colorimetric kit (American Diagnostics Corp., Inc., New York). In this laboratory, the mean BUN level on neutered male and female mice was 30 mg/dl ± 4 SEM for normal male mice and 33 mg/dl ± 4 SEM for normal female mice (23).

Lymphocyte Enumeration and Mitogenic Response

Groups of mice, each group containing 5 NZB/NZW hybrids and 5 C57BL/6 hybrids of each sex were studied on day 8 weeks after birth. No differences were noted in the longevity study, and the results were combined.

Monoclonal Antibodies and Azide

Ig anti-concanavalin A (ConA) (PharMingen, San Diego, CA), 0.04 mg per injection, were given to the groups prior to sacrifice in the last third of pregnancy, after which the groups were sacrificed. The differences were significant by Student's t-test (p < 0.05).

Preparation of Cells

The cell suspensions were prepared by mechanically dissociating spleens in RPMI 1640 (Gibco, Grand Island, NY). The cells were filtered through cotton mesh and washed with RPMI 1640 enriched with 0.5% fetal calf serum (FCS) (Gibco), 1-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 according to the protocol described in the text. The cells were then frozen and thawed in a dry ice-ethanol bath and washed in phosphate-buffered saline (PBS). Cells were stained with monoclonal antibod- ies at saturating concentration (0.2-5 pg/ml) of 3.7 g/ml for 30 min. PE-strep- toavidin was added to tubes containing biotlated antibodies. After washing, 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 h later by flow cytometry analysis.

A FACScan flow cytometer (BectonDickinson) was used under standardized conditions for measurement of light scatter and fluorescence (25). Fluorescence light scatter was collected at 515 ± 10 nm (FITC) and 680 ± 15 nm (PE) in single- or dual-parameter histograms from 10,000 cells with the lymphocyte light scatter gates as defined by forward and perpendicular light scatter signals. Data were expressed as the percent of cells bearing the antigen of interest. When nec- essary, nonspecific staining was subtracted from positive cells.

Lymphocyte Transformation

Mitogenic responses of splenocytes to Concanavalin A (ConA) (PharMingen), 5 × 10^4 (IP/SU) and 0.04 mg per injection, were given to the groups prior to sacrifice in the last third of pregnancy, after which the groups were sacrificed. The differences were significant by Student's t-test (p < 0.05).
Results

Longevity Study of NZB/NZW Offspring

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

Lymphocyte Populations in NZB/NZW Mice

Table 4 displays percentages of cells with surface markers for T-lymphocytes (Thy-1, CD4, and CD8) and B-lymphocytes (IgM). At 8 weeks of age, female NZB/NZW mice from untreated controls had a lower percentage of T-lymphocytes expressing Thy-1.2 compared to NZB/NZW females from sham-treated dams (18 vs 24%, p < 0.01). Treated NZB dams with esculinase was associated with similar reduction in the proportion of CD4+ cells in female offspring (p < 0.65). No significant differences were found between percentages of cells bearing CD3 or IgM in offspring of dams that received esculinase or sham treatment. Data from 16-week-old mice are not shown; at this point, NZB/NZW offspring from untreated or sham-treated dams did not differ with respect to T-lymphocytes or B-lymphocyte markers.

Lymphocyte Populations in C57/DBA2 Mice

Peritoneal cells from 8-week-old C57/DBA2 mice that reacted with the four antibodies of interest are in Table 4. Tenessemic transplanted C57/BLA6 mice 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 mito- gen, ConA, and the B-lymphocyte mito- gen, LPS, are shown in Table 5. At 8 weeks of age, there was a trend for increased T-lymphocyte upregulation in response to both mitogens in females and male offspring of testosterone-treated dams. This increase was significant only for Con A-sensitized 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.

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Table 6. Cell surface antigens on spleen cells from 8-week-old NZB/NZW and C57/B12F1 mice.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Sex of offspring</th>
<th>Minimal treatment</th>
<th>Thiv 1-2</th>
<th>CD4</th>
<th>CD8</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZB/NZW Female</td>
<td>Testosterone</td>
<td>16 ± 2*</td>
<td>16 ± 1*</td>
<td>7 ± 0.5</td>
<td>40 ± 2</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Testosterone</td>
<td>22 ± 2</td>
<td>17 ± 1</td>
<td>32 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shann</td>
<td>22 ± 2</td>
<td>16 ± 1</td>
<td>58 ± 4</td>
<td>54 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57/B12A Male</td>
<td>Testosterone</td>
<td>16 ± 5</td>
<td>13 ± 1</td>
<td>6 ± 1</td>
<td>70 ± 3</td>
<td></td>
</tr>
<tr>
<td>Shann</td>
<td>17 ± 5</td>
<td>13 ± 1</td>
<td>6 ± 1</td>
<td>70 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>16 ± 5</td>
<td>13 ± 1</td>
<td>6 ± 1</td>
<td>69 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shann</td>
<td>27 ± 5</td>
<td>12 ± 1</td>
<td>5 ± 1</td>
<td>68 ± 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Table 7. Response to estrogenic stimulation with ConA and LPS in 8-week-old and 16-week-old NZB/NZW and C57/B12A mice.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Sex of offspring</th>
<th>Minimal treatment</th>
<th>ConA</th>
<th>LPS</th>
<th>ConA</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZB/NZW Female</td>
<td>Testosterone</td>
<td>115 ± 7</td>
<td>45 ± 4</td>
<td>60 ± 4</td>
<td>37 ± 4</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Testosterone</td>
<td>57 ± 10</td>
<td>41 ± 7</td>
<td>63 ± 4</td>
<td>38 ± 2</td>
<td></td>
</tr>
<tr>
<td>Shann</td>
<td>77 ± 13</td>
<td>27 ± 6</td>
<td>60 ± 4</td>
<td>32 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57/B12A Female</td>
<td>Testosterone</td>
<td>57 ± 18</td>
<td>30 ± 7</td>
<td>41 ± 10</td>
<td>36 ± 1</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Testosterone</td>
<td>36 ± 18</td>
<td>47 ± 3</td>
<td>54 ± 15</td>
<td>36 ± 6</td>
<td></td>
</tr>
<tr>
<td>Shann</td>
<td>58 ± 17</td>
<td>48 ± 4</td>
<td>53 ± 16</td>
<td>36 ± 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SEM = 5%. Incorporation of [3H]-thymidine by dividing cells is expressed as mean ± SEM of 4–6 PM (mean ± standard deviation). Testosterone-treated mice versus female offspring of sham-treated dams.

Mitogenic Responses in C57/B12A Mice

In nonsmiminating C57/B12A hybrids, active responses were observed when spleen cells were cultured with either estrogen. Response were not influenced by prenatatal treatment, sex, or age (Table 7).

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 (38,29) 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 dais are treated on days 13 to 18 of gestation with implanted testosterone in a dose that somewhat masculinizes genetica of the female NZB/NZW offspring (16). This treatment results in long-lived male NZB/NZW females that survive for a significantly longer period compared to NZB/NZW male from control dams (16).

This present paper presents measurements of three parameters of active autoimmune disease in females and male NZB/NZW offspring of testosterone-treated and control dams. Anti-DNA, albuminuria, and BUN were assayed serially during the first year of life. These indicators increased as expected at the animals grew older. At 36 weeks of age, female NZB/NZW mice from testosterone-treated dams had active production of anti-DNA (measured by ANA titer), which had been maintained at a low level in the terminal phase of SLE in this group (60% vs 45% in females from sham-manneled mice) (7). Active anti-DNA concentration, a marker of active disease, was not associated with accelerated proliferative disease in the females from hormone-treated dams.

This analysis suggested that prenatal exposure to estrogenic-androgenics was associated with accelerated autoantibody formation in the females. Paradoxically, their lifespans were not shortened compared to same-sex controls. In the corresponding male line, 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 androgens, which may affect the immune system, and males produce immune-suppressing androgens. We are currently examining in these mice even though they had long-standing, active disease that paralleled males from sham-treated dams.

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T-lymphocyte milieu, Con A. The difference in T-lymphocyte production did not persist to 16 weeks of age, and was not noted in NZB/NZW males or C3H/DBA hybrids. Male NZB/NZW mice from treated dams had no changes in lymphocyte sub-
sets, but a significant increase in response to Con A was noted at 8 weeks of age.

Maternal testosterone treatment did have some long-lasting effects on lymphoc-
phoid cell subsets and responses in males. 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 reduced numbers 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 signif-
ificance of this response is not clear; it could reflect an immunological effect of lympho-
phoid growth factors that may or may not be associated with the phenotypic expression of autoimmune disease in NZB/NZW males.

Our evaluations of the immune system in these animals did not identify a single protective factor that could have altered long-term outcome in autoimmune NZB/
NZW mice. Future investigations will focus on identifying changes in the cellular and possible hormonal environments that might improve immunomodulation in NZB/NZW males. The findings that NZB/NZW and male mice have decreased serum estradiol com-
pared to concentrations in male fostered in utero mice, and the existence of autoantibodies strains (16,17,18,20) and that a decrease in serum estradiol during fetal life in NZB/NZW males was associ-
ated with increased longevity, have important implications for studies of envi-
ronmental influences or autoimmune disease. Our findings suggest that environ-
mental 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 recep-
tors and are referred to as environmental estrogen. Man-made environmen-
tal chemicals include pesticides, such as DDT and methoxy-
carb, components of commonly used products such as plastics and soaps, and the epoxy lining of cas, such as ethylphe-
cholines, which are components of commonly used products such as plastics and soaps, and the epoxy lining of cas, such as ethylphe-
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