

## Aberrant Hormone Balance in Fetal Autoimmune NZB/W Mice Following Prenatal Exposure to Testosterone Excess or the Androgen Blocker Flutamide<sup>1</sup>

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### ABSTRACT

F<sub>1</sub> hybrid New Zealand Black (NZB) × New Zealand White (NZW) (NZB/W) mice are hormone-sensitive models of the human disease systemic lupus erythematosus. In this study, NZB/W fetuses produced by pregnant NZB mice were compared with F<sub>1</sub> C57BL/6 × DBA/2 (C57/DBA2) hybrid fetuses produced by nonautoimmune C57BL/6 females. Dams of both strains were treated with testosterone or the androgen blocker flutamide to alter the hormonal environment in late gestation. Hormonal changes in male fetuses carried by treated dams were of interest because hormonal manipulation using either testosterone or flutamide has been shown to increase longevity in male NZB/W offspring. Testosterone-implanted NZB dams developed the expected elevations in circulating maternal testosterone, whereas C57BL/6 dams treated with either testosterone or flutamide had elevated maternal serum testosterone concentrations. The treatment-induced changes in circulating testosterone in NZB dams and C57BL/6 dams were not reflected in serum from 18-day NZB/W or C57/DBA2 fetuses.

Male NZB/W offspring from untreated control NZB dams had unexpectedly high levels of serum estradiol and alpha fetoprotein and relatively low extractable testicular testosterone, compared with nonautoimmune male control fetuses. Maternal testosterone treatments produced a significant decrease in serum estradiol in NZB/W male fetuses, and placental testosterone content was also reduced. Our findings suggest that placental androgen control is regulated differently in the autoimmune NZB-NZB/W vs. the nonautoimmune C57BL/6-C57/DBA2 maternal-placental-fetal unit.

### INTRODUCTION

F<sub>1</sub> hybrid NZB × NZW (NZB/W) mice, which are produced by crossing female New Zealand Black (NZB) mice with male New Zealand White (NZW) mice, spontaneously develop antibodies directed against DNA (anti-DNA) and immune complex nephritis [1, 2]. These mice are accepted as models of the human disease systemic lupus erythematosus. Expression of the autoimmune disorder in NZB/W mice is influenced by gender. Females have accelerated disease and die with renal failure at 10–12 mo of age, whereas males have late-onset disease [3]. Treatment with exogenous steroids in young animals has provided evidence that estrogen stimulates disease [4]. In contrast, androgens have striking protective effects. Male NZB/W mice die prematurely after prepubertal castration [5], and therapy with exogenous testosterone has been shown to improve survival in NZB/W females with established renal disease [6].

Because lupus in NZB/W mice is clearly influenced by steroid hormones, we postulated that altering the hormonal milieu in utero would affect the subsequent course of autoimmune disease in the fetuses. This supposition was in-

vestigated previously with a model in which NZB dams, pregnant with NZB/W fetuses, received testosterone or the androgen receptor blocker flutamide during late gestation. Both drugs were given in dosages sufficient to alter the fetuses' genital morphology without suppressing ovulatory cycles during later life in female offspring [7]. Female NZB/W offspring from flutamide-treated mothers had fewer premature deaths from renal disease than their respective controls. The most striking effects of prenatal hormone treatment, however, were observed in the male offspring. Both maternal treatments—with testosterone and with flutamide—resulted in significant increase of longevity in the male NZB/W offspring [7].

The current report delineates results of an experiment in which groups of NZB dams were treated with the previously established dosages of testosterone or flutamide. Levels of circulating fetal testosterone, estradiol, and alpha fetoprotein (AFP) were determined on Day 18 of gestation. We also assayed testosterone content in the two organs that secrete androgen in the fetus, the placenta and the testes. Activity of aromatase, the enzyme that metabolizes testosterone to estradiol, was assayed in fetal liver.

This study provided the opportunity to compare the hormonal environment during fetal life of autoimmune NZB/W mice and non-autoimmune hybrid C57/DBA2 mice. An additional goal was to gain a better understanding of changes in fetal hormones, induced by maternal treatment, that may influence the late expression of disease in male NZB/W offspring.

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## MATERIALS AND METHODS

### *Animals*

NZB females and NZW males (Jackson Laboratories, Bar Harbor, ME) and C57BL/6 females and DBA/2 males (Harlan Sprague Dawley, Indianapolis, IN) were purchased at the age of 5 wk and maintained in the Research Service of the Harry S. Truman Memorial Veterans' Hospital. Mice were housed in 22 × 33 × 12-cm polycarbonate cages on aspen wood shavings with a pressed block diet (Formulab #5008; Purina Mills, Inc., St. Louis, MO). The animal room was maintained at 22°C with an automatic 12L:12D light cycle. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care, and animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published in 1985 by the Institute for Laboratory Animal Resources, National Research Council-National Academy of Sciences. Females were paired with males at the age of 6 wk and checked daily for vaginal plugs. The appearance of a plug was Day 0.

### *Testosterone Treatment*

One-centimeter implants made from Silastic tubing 602–285, 0.06 in. i.d., 0.125 in. o.d. (Dow Corning, Midland, MI) were filled with 0.75 mg testosterone (Sigma Chemical Co., St. Louis, MO) in 0.02 ml sesame oil as described previously [7]. A capsule was implanted s.c. into a dam on Day 13 of gestation and remained throughout pregnancy. Each mouse was anesthetized with methoxyflurane (Pitman Moore, Mundelein, IL), an implant was inserted s.c. through an incision over the left scapula, and the incision was closed with a surgical clip.

### *Flutamide Treatment*

The antiandrogen flutamide (4'-nitro-3'-trifluoromethylisobutyranilide; Schering, Bloomfield, NJ; 5 mg/day, in 0.4 ml 3:1 v/v sesame oil/isopropyl alcohol vehicle) was injected s.c. between the scapulae of dams for 5 days starting on Day 13 of gestation, with delivery by cesarian section early on the morning of Day 18. The dosage of flutamide was determined previously to reduce the anogenital space (a bioassay for prenatal testosterone action) in male offspring to that of females [7].

### *Controls*

Control NZB and C57BL/6 dams received implants containing 0.02 ml sesame oil on Day 13 of pregnancy, or injections of sesame oil/alcohol vehicle on Days 13–17 of pregnancy. In the initial study in this series, anogenital measurements in male and female offspring of control NZB and C57BL/6 dams were analyzed with attention to the dams' having received either sesame oil implants or injections of vehicle. The two manipulations of the control dams

were not associated with statistically significant differences in hormone concentrations in the dams or anogenital distances in the offspring. Results from the control dams were pooled and treated as one group for all subsequent control analyses [7]. In the current study, the data from control dams that had received "blank" implants or vehicle injections were likewise combined, and data from their offspring were also combined within sex and strain.

### *Derivation of Offspring*

Fetuses were delivered from all dams by cesarean section early on Day 18 of gestation; parturition normally occurs on Day 18. At that time, blood was collected from the dams and fetuses. Serum was separated and stored at –20°C for RIAs. In approximately 10% of fetuses, the volume of serum was not adequate to test for estradiol, testosterone, and AFP. In these instances, serum from two fetuses was pooled within litter, sex, and strain. Because the doses of testosterone and flutamide administered to dams changed anogenital distances in offspring [7], fetal sex was verified by autopsy to identify either ovaries or testes. Steroids in the placenta from each animal, and in one testis in the case of male fetuses, were extracted immediately in preparation for testosterone RIA. Fetal livers were removed, wrapped in foil, and snap-frozen in liquid nitrogen. Frozen tissue was stored at –70°C until assayed for aromatase activity.

### *Testosterone RIA*

Concentrations of testosterone were determined in 25- $\mu$ l aliquots of serum from dams and 15- $\mu$ l aliquots of serum from fetuses according to procedures described by vom Saal et al. [8]. Testes were extracted in 4 ml of ethylacetate:chloroform (80:20) for 36 h and reconstituted in 1 ml assay buffer. Fifteen-microliter aliquots were assayed. Placentae were minced and extracted in 4 ml solvent for 36 h. Tissues were reconstituted in 5 ml assay buffer, and 50- $\mu$ l aliquots were assayed. The minimum detectable concentration of testosterone was 2 pg/tube. Serum samples were run in duplicate in all RIAs. To determine intraassay variability, a pool of mouse serum was diluted, and replicate samples were run at different concentrations in each run. Multiple dilutions of the same pool were used in each run. Variability of results was thereby determined at different points on the binding curve. Inter- and intraassay coefficients of variation for all testosterone assays were 9% and 6%, respectively.

### *Estradiol RIA*

Concentrations of estradiol were determined in 50- $\mu$ l aliquots of sera as described by vom Saal et al. [8]. The minimum detectable concentration of estradiol was 0.25 pg/tube, with inter- and intraassay coefficients of variation of 4% and 7%, respectively.

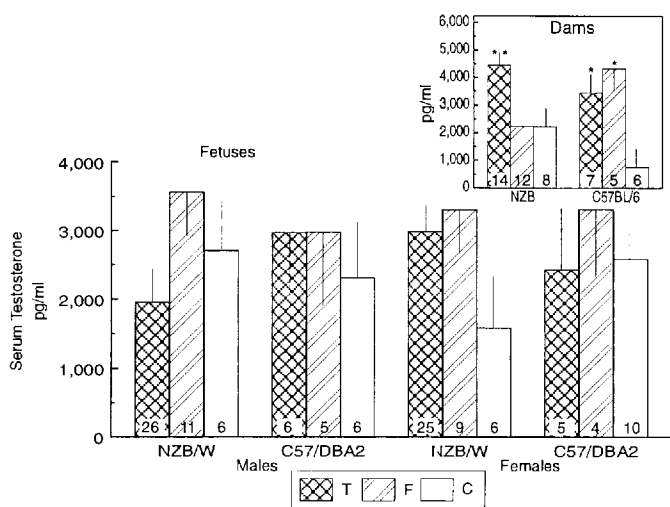


FIG. 1. Serum testosterone concentrations in dams (insert) and fetuses. T, testosterone treatment of dams; F, flutamide treatment of dams; C, sham-treated control dams. Numbers in bars represent number of samples assayed. \* $p < 0.025$ ; \*\* $p < 0.01$  for groups of treated dams vs. control dams of same strain.

### AFP RIA

Sera collected from mice were assayed for AFP as described previously by Keisler et al. [7]. Both fetal and maternal sera were diluted  $1 \times 10^6$  for determination of AFP. The minimum detectable concentration of AFP was 0.1 ng/tube. Inter- and intraassay coefficients of variation were 9% and 5%, respectively.

### Aromatase Activity

The assay for aromatase activity was modified from the methods described by Roselli [9] and Kelce et al. [10]. This technique measures the amount of  $^3\text{H}_2\text{O}$  produced from  $[1\beta\text{-}^3\text{H}]\text{androstenedione}$ , which is proportional to the amount of estrogen formed by the aromatase activity. The specifics of the technique are as follows. Beta NADPH (1 mM), L-glutamine (2 mM), and  $[1\beta\text{-}^3\text{H}]\text{androstenedione}$  (2  $\mu\text{Ci}/\text{sample}$ ; 27.4 Ci/mmol; New England Nuclear, Boston, MA) were added to Eagle's Minimum Essential Medium, which was diluted 1/1 with distilled water and adjusted to pH 7.4. Aliquots of medium (200  $\mu\text{l}$ ) were pipetted into 12  $\times$  75-mm tubes on ice. A tissue slice was added to each tube, and three tubes without tissue were processed as blanks. The tissue was sonicated briefly, and the mixture was incubated with shaking in 95%  $\text{O}_2$ :5%  $\text{CO}_2$  for 1 h at 37°C. Isolation of the  $^3\text{H}_2\text{O}$  product was performed by an extraction technique described by George and Ojeda [11]. One milliliter of chloroform was added to each tube, the tubes were vortexed, and 1.3 ml 10% trichloroacetic acid was added. Tubes were vortexed a second time and centrifuged 5 min at  $1000 \times g$ . One milliliter of the aqueous layer was pipetted into another tube with 1 ml 5% charcoal and 0.5% dextran suspension. After centrifugation, 500  $\mu\text{l}$  of each su-

pernatant was pipetted into a vial containing scintillation fluid and counted. The remaining tissue layer was washed with 5% ethanol, incubated overnight with 0.5 N NaOH at 37°C, and analyzed for protein content by the Lowry assay [12]. Aromatase activity was calculated by use of the following equation:  $6(\text{DPM sample} - \text{DPM blank}) / (2.2 \times 10^6)$  ( $^3\text{H}$  androstenedione specific activity in  $\mu\text{Ci}/\text{nmol}$ ) and expressed as fmol/mg protein/h.

### Statistical Analyses

Analysis of variance followed by planned comparison using the least-significant means test was used to determine group differences for serum testosterone, estradiol, AFP, testicular testosterone, placental testosterone, and liver aromatase (General Linear Model; SAS, Cary, NC, 1985). Treatment of the dam (testosterone, flutamide, or sham treatment), strain, and the interaction of treatment and strain were contained as main effects in the statistical model.

## RESULTS

### Serum Concentrations of Testosterone

**Dams.** Treatment with capsules containing 0.75 mg testosterone resulted in significantly elevated serum testosterone concentrations in both autoimmune (NZB) and non-autoimmune (C57BL/6) dams, compared to respective controls of the same strain: for NZB  $p < 0.01$ ; for C57BL/6,  $p < 0.025$  (Fig. 1). These data confirmed transfer of testosterone from Silastic implants to the maternal circulation of pregnant females, through use of a protocol that has been shown to masculinize genitalia of female offspring [7]. Maternal serum testosterone concentrations were not affected by flutamide treatment in NZB dams. Pregnant C57BL/6 mice, however, responded to flutamide with significantly increased concentrations of serum testosterone ( $p$  vs. controls  $< 0.025$ ).

**Fetuses.** In male NZB/W fetuses delivered from NZB dams, mean serum testosterone concentrations were not different from those in NZB/W controls as a result of treatment of the dam with either testosterone or flutamide (Fig. 1). Treatment effects were also absent in male C57/DBA2 fetuses from C57BL/6 dams that received either drug. In the female fetuses, treating NZB or C57BL/6 dams with testosterone or flutamide did not influence mean testosterone concentrations.

### Serum Concentrations of Estradiol

**Dams.** NZB dams treated with testosterone had a serum estradiol concentration of (mean  $\pm$  SEM)  $115 \pm 26$  pg/ml compared to  $49 \pm 30$  pg/ml in corresponding controls ( $p = 0.14$ ); NZB dams treated with flutamide had a mean serum estradiol concentration of  $94 \pm 30$  pg/ml (Fig. 2). In C57BL/6 dams, testosterone treatment did not alter circu-

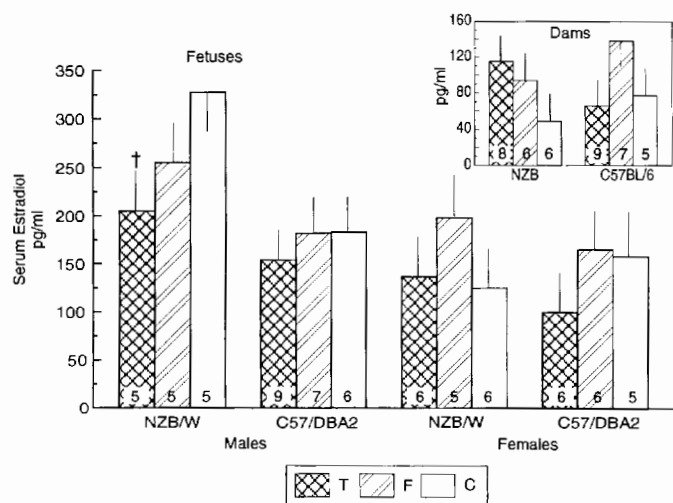


FIG. 2. Estradiol in dam serum (insert) and fetal serum. T, testosterone treatment of dams; F, flutamide treatment of dams; C, sham-treated control dams. † $p < 0.05$  for male NZB/W fetuses compared to respective controls.

lating maternal estradiol concentrations; however, flutamide treatment did result in serum estradiol that was twice the concentration of that in corresponding controls ( $138 \pm 28$  pg/ml versus  $77 \pm 33$  pg/ml, respectively), although again the difference was not statistically significant.

**Fetuses.** Estradiol concentrations were greater overall in the serum of all NZB/W fetuses compared with all C57/DBA2 fetuses ( $262 \pm 24$  pg/ml vs.  $173 \pm 20$  pg/ml,  $p < 0.01$ ), and the mean circulating estradiol concentration in control NZB/W males was greater than that in control C57/DBA2 males ( $p < 0.01$ ). Mean circulating estradiol was also significantly greater in control NZB/W male ( $327 \pm 42$  pg/ml) than in control female ( $125 \pm 44$  pg/ml) fetuses ( $p < 0.01$ ).

Male NZB/W fetuses from testosterone-treated dams had a lower mean serum estradiol concentration (vs. NZB/W male controls,  $p < 0.05$ ), but flutamide treatment of the dams did not significantly affect estradiol in NZB/W male fetuses (Fig. 2). Treating C57BL/6 dams with testosterone or flutamide did not significantly alter circulating estradiol in the male C57/DBA2 fetuses when compared with control C57/DBA2 male fetuses, nor did either treatment affect mean estradiol in female NZB/W or C57/DBA2 fetuses.

#### Serum Concentrations of AFP

**Dams.** AFP concentration in NZB dams receiving testosterone was  $400 \pm 12$   $\mu$ g/ml compared to  $59 \pm 14$   $\mu$ g/ml in corresponding control dams,  $p < 0.01$  (Fig. 3). In flutamide-treated NZB dams, circulating AFP was  $127 \pm 13$   $\mu$ g/ml ( $p < 0.05$  vs. controls). AFP concentrations in C57BL/6 dams treated with testosterone or flutamide were  $113 \pm 12$   $\mu$ g/ml and  $112 \pm 13$   $\mu$ g/ml, respectively, compared to  $61 \pm 13$   $\mu$ g/ml in control C57BL/6 dams ( $p < 0.01$  for both comparisons).

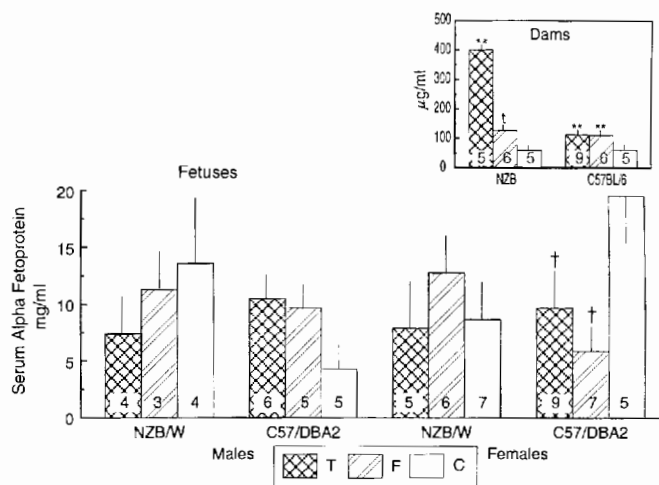


FIG. 3. Serum AFP in dams (insert) and fetuses. T, testosterone treatment of dams; F, flutamide treatment of dams; C, sham-treated control dams. \*\* $p < 0.01$ ; † $p < 0.05$  for groups of treated dams vs. control dams within strain. † $p < 0.05$  for female C57/DBA2 fetuses from testosterone-treated or flutamide-treated dams vs. female C57/DBA2 fetuses from control dams.

**Fetuses.** For male fetuses carried by control dams, Figure 3 shows elevated mean serum AFP in male NZB/W fetuses compared to levels in corresponding male C57/DBA2 fetuses ( $p < 0.02$ ). In fetuses from control dams, control NZB/W female fetuses had significantly lower concentrations of serum AFP ( $9 \pm 3$  mg/ml) than did control C57/DBA2 female fetuses ( $20 \pm 4$  mg/ml),  $p < 0.05$ .

Responses to the maternal treatments were influenced by strain in male offspring. NZB/W males had no significant changes in AFP when their dams received testosterone or flutamide; however, NZB/W males whose dams were treated with testosterone did show somewhat lower levels of AFP ( $7 \pm 3$  mg/ml) relative to controls ( $13 \pm 3$  mg/ml). In contrast, although concentrations of AFP tended to be greater in male C57/DBA2 offspring of dams treated with testosterone or flutamide, the differences were not significant ( $p = 0.07$ ).

In female NZB/W fetuses, AFP concentrations were not affected by exogenous hormonal manipulation of the dam. In contrast, treating female offspring from C57BL/6 dams with testosterone or flutamide resulted in significant suppression of the relatively high levels of circulating AFP, as compared to levels in female C57/DBA2 controls ( $p < 0.05$  for each group).

#### Placental Testosterone

Figure 4 (left panel) depicts testosterone extracted from placental tissue on Day 18 of gestation. In the placentae of male fetuses, response to hormonal manipulation of the dam differed by strain. Male NZB/W fetuses, delivered from groups of NZB dams treated with testosterone or flutamide, had in common a striking suppression of placental testos-

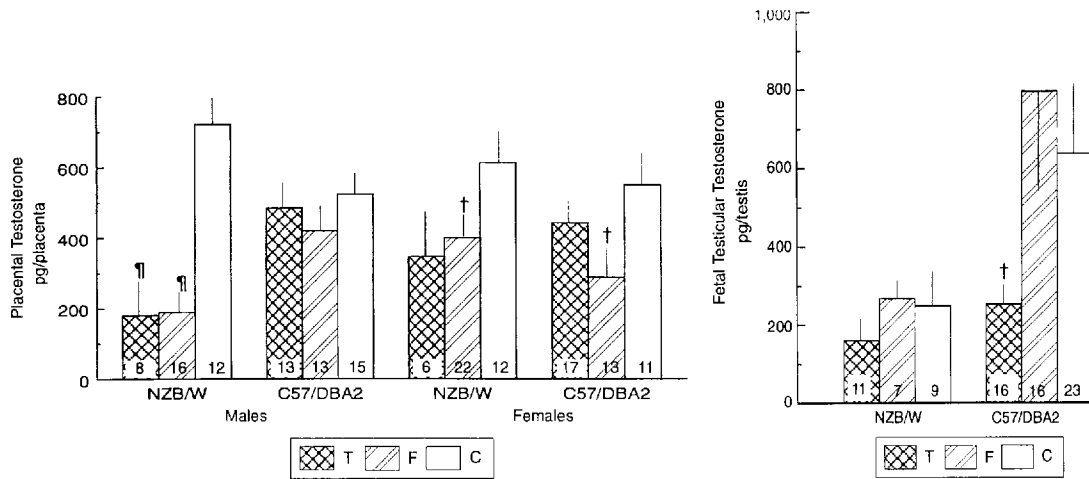


FIG. 4. **Left panel:** placental testosterone. † $p < 0.05$ ; †† $p < 0.0001$  for groups of fetuses from treated dams, compared to same-sex fetuses of same strain from control dams. **Right panel:** fetal testicular testosterone. One testis was studied from each animal. † $p < 0.05$  for fetuses from testosterone-treated C57BL/6 dams vs. fetuses from control C57BL/6 dams. T, testosterone treatment of dams; F, flutamide treatment of dams; C, sham-treated control dams.

terone content (in each group,  $p < 0.0001$  compared to male NZB/W controls). In contrast, treatment of C57BL/6 dams with either drug did not alter testosterone content in the placentae of C57/DBA2 males.

In female fetuses, strain of the dam did not influence mean placental testosterone; rather, placental testosterone content was treatment-dependent. This was reflected in a decrease of placental testosterone content in the placentae of NZB/W female fetuses from testosterone-treated NZB dams (vs. female control fetuses,  $p = 0.08$ ) and in placentae of fetuses from flutamide-treated NZB dams ( $p < 0.05$ ). In female C57/DBA2 offspring, fetuses from flutamide-treated dams had less placental testosterone compared with that in corresponding female controls ( $p < 0.05$ ).

**Testicular Testosterone**

Concentrations of testosterone in extracts of testicular tissue from male offspring are presented in Figure 4 (right

panel). Overall, concentrations of testicular testosterone in all NZB/W males (combined mean 225 pg/testis) was significantly lower than values in all C57/DBA2 males (combined mean 536 pg/testis);  $p < 0.01$ . Hormonal manipulation of the dam did not alter testicular testosterone in NZB/W fetuses. Treating C57BL/6 dams with testosterone, however, produced hybrid C57/DBA2 fetuses with significant suppression of testicular testosterone ( $p < 0.05$  vs. controls) while flutamide treatment had no effect on testicular testosterone content.

**Liver Aromatase Activity**

In male control NZB/W fetuses, mean baseline aromatase activity was quite low ( $0.20 \pm 0.30$  fmol/mg protein/h) compared to activity in control female NZB/W fetuses ( $1.80 \pm 0.30$  fmol/mg protein/h);  $p < 0.01$ ) (Fig. 5). Unlike the finding for NZB/W fetuses, control male and female C57/DBA2 fetuses did not differ in their mean aromatase activity. Control male NZB/W fetuses showed significantly ( $p < 0.05$ ) lower mean aromatase activity than did control male C57/DBA2 fetuses, while control female NZB/W fetuses showed significantly ( $p < 0.05$ ) higher mean aromatase activity than did control female C57/DBA2 fetuses.

Aromatase activity was influenced significantly in autoimmune NZB/W hybrids by treatment of the NZB dam. Testosterone treatment of NZB dams resulted in a significant 6-fold elevation of mean hepatic aromatase activity (vs. control NZB/W males,  $p < 0.01$ ). A 4-fold increase of aromatase activity was detected in male offspring of flutamide-treated NZB/W dams. Female fetuses from flutamide-treated NZB dams had lower mean aromatase activity (vs. corresponding NZB/W controls,  $p < 0.01$ ), but females from testosterone-treated dams were not significantly affected. Mean hepatic aromatase activities in male and female C57/

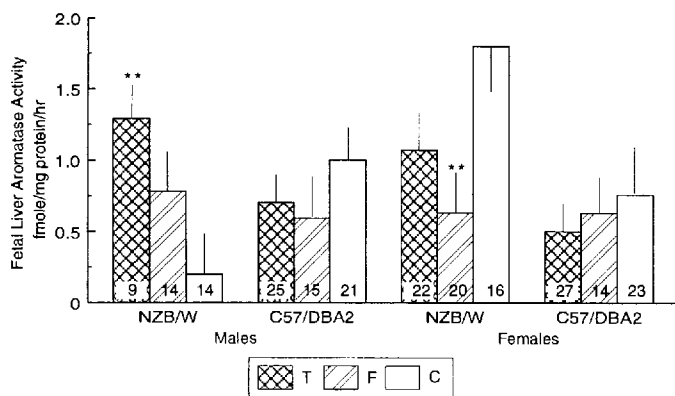


FIG. 5. Fetal liver aromatase activity. T, testosterone treatment of dams; F, flutamide treatment of dams; C, sham-treated control dams. \*\* $p < 0.01$  for male and female NZB/W offspring of flutamide-treated dams vs. same-sex NZB/W controls.

DBA2 fetuses were not affected by either maternal treatment, and values did not differ significantly from those in the respective controls.

### DISCUSSION

One goal of the current study was to investigate the late gestational environment in a murine model that spontaneously develops immune-mediated disease responsive to gonadal hormones. In prior studies with CF-1 mice, we found that female fetuses had significantly higher circulating estradiol than did male fetuses [13]. The current study showed that the concentration of serum estradiol in NZB/W female fetuses was similar to that reported in CF-1 female fetuses [13]. It was therefore unexpected to find that control male NZB/W fetuses had over two times greater serum concentrations of estradiol (327 pg/ml) relative to their female siblings (125 pg/ml). Because unmanipulated NZB/W males are always expected to have a longer life span than NZB/W females, these findings were opposite to the predicted relationship between fetal estradiol levels and severity of autoimmune disease. However, males and females are also exposed to different hormonal environments throughout postnatal life, and comparisons between longevity in males and females gonadectomized at birth would be needed to directly assess the isolated role of fetal hormones on longevity.

We showed previously that manipulating the prenatal hormonal environment of NZB/W mice resulted in a significant increase in lifespan in male offspring [7]. A major hormonal change due to administering testosterone to pregnant NZB dams was a decrease in the concentration of estradiol in the serum of male NZB/W fetuses. In contrast to the findings in the male fetuses, hormonal manipulations of the dam had only a small effect on prolonging longevity in female offspring [7]. Of interest, serum estradiol in female NZB/W fetuses was not altered by either maternal treatment.

The very high concentrations of circulating estradiol in male NZB/W fetuses were reduced by approximately 75 pg/ml as a result of maternal treatment with flutamide, and by 125 pg/ml through maternal treatment with testosterone. It is proposed that a change of 75–125 pg/ml in circulating estradiol is sufficient to change tissue responses to estradiol. This assumption is based upon the observation that implanting a Silastic capsule containing estradiol into pregnant CF-1 mice led to 100 pg/ml increases of circulating estradiol in the CF-1 mouse fetuses. This treatment of the dam dramatically altered the functioning of the prostate during subsequent adult life in the male offspring. Increased numbers of cells in the prostate, and increased androgen receptors per cell were observed [14]. Such an effect could also conceivably "prime" cells in the immune system to have altered responsiveness to immune-modulating hormones, with long-term effects leading to delayed death from autoimmune disease.

The decrease in estradiol we observed in NZB/W male fetuses was in the concentration of total estradiol in the blood, which includes estradiol not bound to plasma proteins (the free, biologically active fraction) as well as estradiol weakly bound to albumin and avidly bound to the plasma estrogen binding glycoprotein, AFP. We thus examined concentrations of plasma AFP in fetuses in addition to estradiol, since changes in the concentration of AFP can alter the concentration of estradiol that is free to pass into cells from the blood. In other studies, the free fraction of estradiol in male mouse fetuses was linearly related to the total plasma concentration of estradiol [15]. Overall, there was fairly good correspondence between plasma AFP and estradiol concentrations in fetuses from both strains. For example, control male NZB/W fetuses had elevated plasma estradiol as well as AFP when compared with testosterone-exposed male NZB/W male fetuses. This finding suggests that the difference in total circulating estradiol might not have been reflected in a corresponding difference in free estradiol, although this remains to be determined. However, it is known that there are different isoforms of AFP with regard to binding affinity for estradiol [16], and our immunoassay does not discriminate between AFP isoforms that bind estradiol with high versus low affinity.

We examined tissue content of testosterone as a key to the presence of inherent differences that may have altered prenatal steroid concentrations and contributed to alterations of the immune system. Steroids are not stored in the cells that produce them. Most evidence suggests that the rate of secretion of steroid hormones reflects the rate of synthesis and is thus correlated with organ content [17, 18].

In the fetal male mouse, one source of testosterone is the testes. Testosterone content increases in testicular tissue by Day 13 of gestation, and peak concentrations are reached on Day 17 [19]. A striking finding in this study was the low gonadal testosterone concentrations in NZB/W fetuses compared to nonautoimmune C57/DBA2 fetuses. Furthermore, maternal treatment did not inhibit extractable testicular testosterone in the lupus-prone offspring. These findings are consistent with a primary defect, in which normal concentrations of androgens could not be produced by testicular tissue in the NZB/W fetus. It is also possible that the stimulus for production of testosterone in fetal life was defective, resulting in decreased pituitary production of LH or diminished LH-like activity in placental fluid [20–23].

The other known source of testosterone production in fetal mice is the placenta. Rat and mouse placentae secrete progesterone and androgen, but not estrogen [17, 24, 25]. In the current study, control NZB/W fetuses had placentae that actively produced testosterone. Treating the NZB dams produced dramatic effects. Extractable placental testosterone (and by inference, synthesis of testosterone) was decreased modestly in placentae of NZB/W females delivered from NZB dams treated with either testosterone or flutam-

ide. In male NZB/W pups from the same dams, placental testosterone was decreased markedly.

Regulation of placental testosterone production is not understood. Our experiments, however, suggest that control mechanisms differ between the autoimmune NZB-NZB/W maternal-fetal-placental unit and a nonautoimmune dam and her fetuses. In the NZB system, high concentrations of testosterone in maternal blood may have exerted a negative feedback effect on placental testosterone that was not observed in the nonautoimmune C57/DBA2 males. The observation that placental synthesis of testosterone showed identical responses to both testosterone and flutamide treatment (Fig. 4) suggests that flutamide may have acted as a weak androgen rather than a pure androgen antagonist.

The assumption that flutamide had weak androgenic effects is supported by the observation that administration of testosterone or flutamide produced generally similar findings with regard to both fetal hormone levels and subsequent longevity. In general, the magnitude of effects due to flutamide was smaller than that of effects due to testosterone. Flutamide may be similar to other synthetic steroid receptor antagonists that act as an antagonist in one tissue but are agonists in other tissues. A well-known example is tamoxifen [26]. In the case of flutamide, the antagonist action would be demonstrated in androgen-sensitive tissues such as the seminal vesicles [7], but the agonist activity would be shown by feedback suppression of testosterone production in the placenta.

We measured the activity of the enzyme aromatase, which synthesizes estradiol from androgen, to determine whether this enzyme was changed by maternal treatment with either testosterone or flutamide. In general, groups of NZB/W fetuses with the highest liver aromatase activity had the lowest levels of circulating estradiol. For example, NZB/W male fetuses carried by mothers treated with testosterone had significantly higher liver aromatase activity (and significantly lower circulating estradiol) relative to control NZB/W male fetuses. One possible explanation for these findings is that the liver is not the major source of androgen conversion to circulating estradiol in these fetuses. It is also possible that estradiol may have an inhibitory effect on liver aromatase activity.

Our findings suggest that changes in longevity of male NZB/W mice whose mothers were administered testosterone or flutamide during pregnancy is, at least in part, mediated by a decrease in circulating estradiol during fetal life. This finding contrasts with the accepted notion that hormonal effects on NZB/W mouse disease occur at the age when the animals reach sexual maturity. Estrogen appears to be immunostimulatory. Although castrated NZB/W females do not have altered longevity [27], implants of 17 $\beta$ -estradiol have been reported to stimulate formation of autoantibodies in male NZB/W mice [5]. In contrast, testosterone has suppressive effects. Castration of NZB/W

males delays onset of autoimmune disease, and continuous androgen treatment of castrated males prevents expression of lupus [6, 28, 29].

In conclusion, the experiments reported here support the argument that on Day 18 of gestation, male NZB/W fetuses have high levels of circulating estradiol associated with variant control of placental testosterone production and low extractable testicular testosterone. It is recognized that the stress associated with impending parturition could have altered concentrations of testosterone and estradiol [30], and that our findings might have differed from the actual status of late-gestation fetuses. The relevance of the murine model to human disease, however, mandates further study of changes in the prenatal steroid environment in the male NZB/W fetus, in order to define contributions of prenatal and post-mature hormone production to the subsequent course of autoimmune disease.

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