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Hormonal Manipulation of the Prenatal Environment Alters Reproductive Morphology and Increases Longevity in Autoimmune NZB/W Mice[1]

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ABSTRACT

Steroid hormones, which affect development of reproductive traits, alter immune responses in rodents and appear to control severity of disease in F_1 hybrid NZB/W mice, an animal model of systemic lupus erythematosus. We tested the hypothesis that exposure of NZB/W fetuses to altered hormonal environments would influence subsequent expression of autoimmune renal disease and affect longevity. NZB females, pregnant with NZB/W fetuses, were treated from Days 13–18 of gestation with testosterone or the antiandrogen, flutamide. Similar treatments were carried out in C57BL/6 dams mated to DBA/2 males to permit comparison with nonautoimmune hybrid mice. Serum concentrations of testosterone were greater in testosterone-implanted dams of both strains, but concentrations of estradiol were greater only in C57BL/6 dams treated with flutamide. Alpha fetoprotein (AFP), which binds estrogen and modulates immune responsiveness, was greater in serum from both groups of testosterone-treated dams, while flutamide treatment increased serum AFP only in NZB dams. We conclude that factors governing circulating estradiol and AFP differed in pregnant NZB and C57BL/6 females. Morphological analyses confirmed effects of hormonal manipulation on the developing fetuses. Testosterone implants resulted in female offspring with greater anogenital spaces, and treatment of dams with flutamide eliminated the expected difference between anogenital spaces in females and males.

Effects of altered prenatal hormonal environments on immune-mediated disease in NZB/W offspring were examined in a longevity study. Early deaths were delayed in NZB/W females produced by flutamide-treated dams. An unexpected result was observed in NZB/W males. Male offspring from both testosterone- and flutamide-treated mothers lived longer than males from control dams. This paradox suggested that a characteristic shared by both groups of treated NZB dams had similar effects on the developing fetuses. It is proposed that elevated concentrations of AFP modulated the course of autoimmune disease and contributed to increased longevity in NZB/W offspring of treated dams.

INTRODUCTION

Phenotypic differences between males and females are influenced by the environment in which the fetus develops, and exposure to gonadal hormones in prenatal life profoundly influences subsequent behavior and reproductive function [1–5]. Testosterone, which affects many biological systems, is a potent determinant of morphological changes during fetal development [6]. Testosterone induces the Wolffian ducts to persist and influences development of external genitalia of males and females [7]. Effects of prenatal exposure to testosterone are observed long after birth in females of litter-bearing species such as the rat and the mouse. For example, the intensity of aggressive behavior and length of estrous cycles in adulthood are determined, in part, by exposure of the developing female to varying concentrations of testosterone [4, 8].

In rodents, prenatal exposure to high concentrations of estrogen can also exert masculinizing influences on centers in the brain that control reproductive functions such as ovulation. Alpha fetoprotein (AFP), a serum protein that binds strongly and specifically to estradiol, is produced in the liver and yolk sac of fetal rats and mice [9]. It has been proposed that the binding of AFP to estradiol in plasma prevents circulating concentrations of estradiol from entering tissues and permits normal development of neural mechanisms controlling reproduction in females.

Androgens, estrogens, and AFP, which have important roles in sexual development, may influence the developing immune system. Steroid hormones have been shown to modulate immune responses in adult rodents [10]. In the gravid mouse, estradiol is elevated in maternal serum from Days 16–17 of gestation until parturition [11]. Concentrations of testosterone peak on Day 9, and a second increase occurs on Days 14–17 [12]. Fetal lymphocytes begin to mature on Day 13 of gestation, and subsequent migration and differentiation give rise to lymphocyte subpopulations that are immunologically active during the latter part of fetal development and in the adult animal [13, 14]. Therefore, precipitous increases in concentrations of hormones in the fetus during gestation coincide with periods of accelerated immunologic development.

The hypothesis that circulating hormones influence fetal morphology, and may have effects on developing lympho-

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cytes, led us to design a study in which fetal mice predisposed to develop lethal immune-mediated disease were exposed to altered endocrine environments. Autoimmune New Zealand Black (NZB) dams crossed with New Zealand White (NZW) males produce F_1 hybrid NZB/W offspring that spontaneously develop disease resembling systemic lupus erythematosus. NZB/W hybrids have autoantibodies directed against DNA [15], and the females die prematurely at 10 mo of age from immune complex glomerulonephritis, vasculitis, and renal failure. The males are expected to live to 16 mo of age [16]. NZB/W mice are of particular interest because expression of their disease is modified by gonadal hormones [17]. When males are castrated, longevity is reduced to that of females. If castrated males receive exogenous testosterone, life span increases to that of intact male controls. In contrast, treatment with estradiol stimulates production of anti-DNA antibodies and results in premature death in mice of both sexes [17–19]. In the current study, pregnant NZB dams were treated with exogenous testosterone or with flutamide, an androgen receptor blocker [20]. Maternal responses to treatments were investigated by assaying serum concentrations of testosterone, estradiol, and AFP, and by morphologic examination of reproductive tissues in offspring of treated dams to verify that changes were induced by abnormal endocrine environments. Results were compared with C57BL/6 dams crossed with DBA/2 males. These mice, and their F_1 hybrid C57/DBA2 offspring, do not develop autoimmune disease. We postulated that treating NZB dams with testosterone or with flutamide would alter the developing NZB/W fetuses, leading to modulation of immune-mediated disease and altered longevity in the adult NZB/W mice.

MATERIALS AND METHODS

Animals

Animals used in these experiments were maintained in facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. The experiments reported here were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Department of Health, Education, and Welfare Publ. No. (National Institutes of Health) 80-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD.

Female NZB and male NZW mice were purchased from Jackson Laboratories (Bar Harbor, ME) to produce autoimmune F_1 hybrid NZB × NZW (NZB/W) offspring. For comparison, female C57BL/6 mice and male DBA/2 mice were purchased from Harlan-Sprague Dawley (Indianapolis, IN) to produce nonautoimmune C57BL/6 × DBA/2 (C57/DBA2) offspring. All mice were obtained at 5 wk of age and maintained in conventional housing in timer-regulated 12L:12D cycles in the Research Service of the VA Medical Center, Columbia, MO. Female mice were paired with males at 6 wk of age. Females were checked daily for vaginal plugs (Day 0 of pregnancy). All pups were delivered via cesarean section on Day 18 of gestation. When pups were breathing normally, anogenital spaces were measured under a dissecting microscope with calipers and a micrometer lens accurate to 0.05 mm to determine effectiveness of treatments. Where appropriate, healthy pups were fostered to lactating CF-1 mothers that had delivered normally in the preceding 24 h. Pups were weaned at 21 days of age and housed 5 per cage with mice of the same sex, from the same hybrid cross. Mice were then assigned at random to experimental groups.

Treatment of Dams

Testosterone. Silastic implants (I.D. 1.52 mm, O.D. 3.18 mm, 1 cm long) containing 0.75 mg testosterone in 0.02 ml sesame oil were placed subcutaneously in dams on Day 13 of gestation. The dose was chosen on the basis of preliminary studies utilizing implants containing testosterone (range = 0.1–5.0 mg) in CF-1 mice. The 0.75-mg dose was the greatest amount that increased (by about 25%) anogenital space (length separating the anus and the genital papilla) [21] without inhibiting opening of the vagina at puberty.

Flutamide. Dams received daily subcutaneous injections of 5 mg flutamide in a mixture of 0.03 ml sesame oil and 0.01 ml alcohol on Days 13–18 of gestation. This dose was chosen on the basis of a pilot study examining 5 log doses of flutamide (F.S. vom Saal, unpublished observation). A daily injection of 5 mg flutamide, given to the dam during the last third of pregnancy, was the lowest dose of flutamide that reduced the anogenital space in male offspring comparable to that of the females.

Sham treatment. Control dams received implants containing sesame oil or daily injections of vehicle. On Day 18 of pregnancy, cesarean sections were performed, the dams were killed, and maternal blood was collected for hormone assays and kept on ice until centrifugation. Sera were stored at −30°C until assayed.

Radioimmunoassays

Testosterone. Concentrations of testosterone were determined in 25-μl aliquots of serum using procedures described by vom Saal, et al. [22]. The minimum detectable concentration of testosterone was 2 pg/tube. Inter- and intraassay coefficients of variation were 10% and 3%, respectively.

Estradiol. Concentrations of estradiol were determined in 50-μl aliquots of serum as described by vom Saal et al. [22]. The minimum detectable concentration of estradiol was 0.25 pg/tube. Inter- and intraassay coefficients of variation were 7%.

AFP. An assay adapted from the procedure of Rouslahti and Seppala [23] was used to determine serum concentrations of mouse AFP. Serum was diluted 1×10^6 for deter-

mination of concentrations of AFP. Mouse AFP (Calbiochem, La Jolla, CA) was iodinated by a modification of the chloramine-T procedure described by Greenwood and Hunter [24]. The specific activity attained was 60.9 μCi/μg protein. First antibody (rabbit antimouse; ICN, Lisle, IL, diluted 1:100 000, was added (100 μl) to tubes and incubated with serum for 1 h at 4°C. ^{125}I-AFP (3 ng/ml) was then added to all tubes (100 μl) and incubated with first antibody and sample for 18 h. Second antibody (1:15; 200 μl; sheep anti-rabbit gamma globulin) was then added. After 90 min, tubes were centrifuged for 45 min and suprenatants were aspirated. Pellets were counted as described previously for the testosterone assay. The minimum detectable concentration of AFP was 0.1 ng/tube. Inter- and intraassay coefficients of variation were 10% and 5%, respectively.

Seminal Vesicle Weights

At 16 wk of age, male offspring of testosterone-treated, flutamide-treated, and sham-treated NZB and C57BL/6 mothers were weighed, and fresh blood was collected at 1300 h. Seminal vesicles were removed and weighed.

Ovarian Transplants

Preliminary studies provided evidence that implantation of a capsule containing 0.75 mg testosterone into CF-1 dams on Day 13 of gestation increased anogenital space and permitted vaginal opening in female offspring. Hybrid females produced by testosterone-treated NZB and C57BL/6 dams had the expected increase in anogenital space but exhibited imperforate vaginas. Transplantation of ovarian tissue to the eye was therefore performed to determine if the dose of 0.75 mg testosterone in the dam altered the female offspring to the point of suppressing the capacity to ovulate [25].

Groups of 3–6 NZB/W and C57/DBA2 female offspring from testosterone-treated or sham-operated mothers had hysterectomies at 12 wk of age (described below). Four weeks later, unilateral ovariectomies were performed under sodium pentobarbital anesthesia (90 mg/kg body weight, i.p.), and each ovary was minced separately in a petri dish containing sterile saline at 4°C. An ovarian autotransplant of a piece less than 0.5 mm in diameter was made behind the cornea of the donor. Females receiving grafts were housed with males to induce regular ovarian cycles, although the mice were separated by wire mesh partitions to prevent mating. Ovarian transplants were examined daily under a dissecting microscope. Observations of preovulatory follicles and subsequent formation of corpora lutea, followed by corpora lutea regression and a second ovulation, were considered evidence that the female was exhibiting ovulatory cycles.

Longevity Protocol

Animals. NZB/W offspring of testosterone-treated mothers, flutamide-treated mothers, and controls were placed in a longevity study and examined daily for signs of disease.

Vaginal opening did not occur in female offspring of testosterone-treated dams by 12 wk of age and all of these offspring had hysterectomies to prevent uterine infections, which often occur due to lack of uterine drainage. The uterine horns and the cervix were removed anterior to the vagina through a mid-ventral incision under methoxyflurane anesthesia. Ovaries were carefully left intact. To determine if hysterectomy affected longevity, 10 control females from sham-implanted mothers were hysterectomized. The course of autoimmune disease in these mice did not differ from other controls, and their longevity data were pooled with other female NZB/W offspring of sham-operated dams.

Determination of Cause of Death. To determine if anti-DNA antibodies and parameters of renal disease were affected by endocrine treatment of dams, mice were bled from the orbital plexus and urine was collected at 3, 6, 9, and 12 mo of age, as well as at spontaneous death [Keisler L.W. Caldwell C.W. vom Saal F.S., Kier A.B., Walker S.E., unpublished results].

Mice were examined daily for signs of disease. Animals were killed and necropsied when they developed evidence of advanced renal failure (loss of muscle mass, lethargy, ascites, labored breathing, rough fur) or when a mass appeared indicating the presence of a neoplasm. Complete necropsies were performed to determine causes of death, and sera were collected to be assayed for anti-DNA antibodies [26] and blood urea nitrogen (BUN) [27]. Urine was tested for albuminuria on Albustix (Ames Co., Elkhart, IN). Hematoxylin and eosin-stained sections of lung, heart, liver, gonads, thymus, spleen, and lymph nodes were examined by light microscopy. Severity of glomerulonephritis was assessed by counting numbers of specified abnormalities in 20 glomeruli in a cross section of each kidney [28]. Vasculitis was identified when the arterial wall had loss of architecture and narrowing of the lumen [29]. It was determined that the cause of death was autoimmune disease if mice had histological evidence of proliferative glomerulonephritis with renal lesion scores ≥ 35 and/or vasculitis in tissue obtained at necropsy. This determination was supported, in almost every instance, by concomitant elevated serum anti-DNA (DNA binding ≥ 20%), increased serum BUN (≥40 mg/dl), and/or increased proteinuria (300–≥2 000 mg/dl).

Statistical Analyses

Analysis of variance and least-significant means comparisons were used to determine group differences in dependent variables in each experiment (General Linear Model: SAS, Cary, NC, 1985). Seminal vesicle weights were compared among groups within hybrid after statistical adjustment for body weight using analysis of covariance [30]. Longevity data were analyzed as a 3 by 2 factorial arrangement of treatment (analysis of variance). The statistical model contained the effects of treatment of the dam (testosterone,

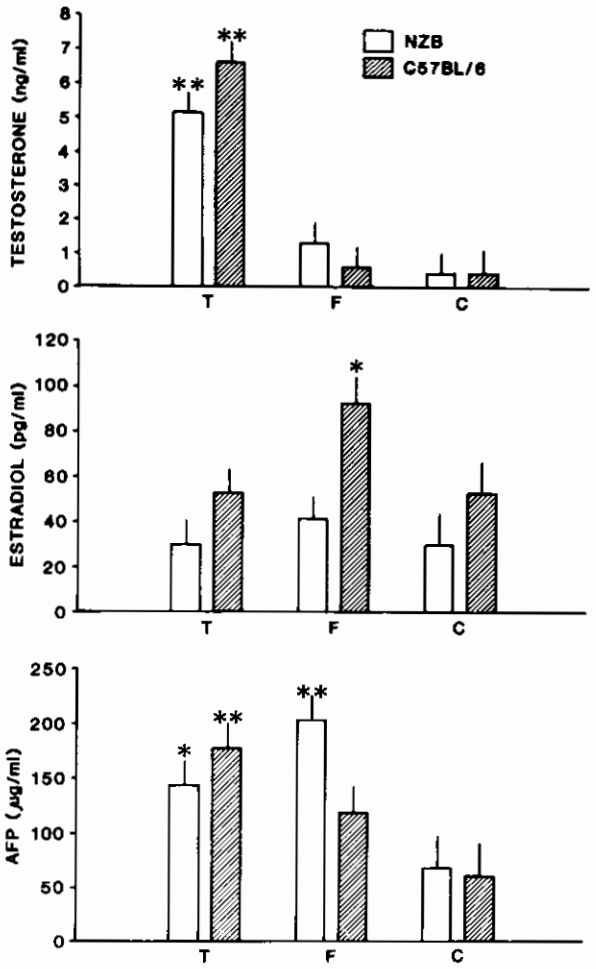

FIG. 1. At 1300 h on Day 18 of pregnancy, concentrations of testosterone (top panel), estradiol (middle panel), and AFP (lower panel) were assayed. The NZB/W offspring of NZB dams predictably developed autoimmune disease. Results were compared with C57BL/6 dams carrying nonautoimmune C57BL/DBA2 fetuses. From Days 13–18 of pregnancy, females received implanted testosterone (T) 0.75 mg (15 NZB, 8 C57BL/6), injections of flutamide (F) 5 mg/day (15 NZB, 8 C57BL/6), or empty capsules/vehicle (C) (20 NZB, 4 C57BL/6). Values are expressed as mean ± SEM. * = $p < 0.05$ vs. controls of the same strain; ** = $p < 0.01$ vs. controls of the same strain.

flutamide, or sham treatment), sex, and the interaction of treatment and sex. Mean differences were ascertained using the LS means test on SAS. Median ages at death were compared using the median test [31].

RESULTS

Maternal Concentrations of Testosterone, Estradiol, and AFP

Serum concentration of testosterone. Implantation of capsules containing 0.75 mg testosterone resulted in greater serum concentrations of testosterone in NZB dams and in C57BL/6 dams (Fig. 1). Concentrations of testosterone in both strains were greater compared to corresponding control dams (for testosterone-treated vs. control groups within each strain, $p < 0.01$; Fig. 1). these determinations verified transfer of the hormone from Silastic capsules to maternal blood. In dams treated with flutamide, it was expected that binding of this androgen blocker to androgen receptors would make the pituitary or placentae unresponsive to the possible inhibitory effects of androgens and result in increased concentration of circulating testosterone. This effect has been observed in intact adult males [32], but in the current studies flutamide treatment did not induce significant elevation of serum testosterone in NZB or C57BL/6 dams. In both flutamide-treated and control dams, mean concentrations of testosterone remained below 2 ng/ml. This is more than 10 times greater than that found in cycling adult mice, but it is normal for late pregnancy [4].

Serum concentrations of estradiol. On Day 18 of gestation, concentrations of estradiol in NZB dams were unaffected by treatment with testosterone or flutamide, and there was a consistent trend to diminished serum concentrations of estradiol in all three groups of treated and control NZB dams compared to the "normal" C57BL/6 strain. In contrast, C57BL/6 dams responded to injected flutamide with elevated serum concentration of estradiol that was 2-fold greater than the value for C57BL/6 controls ($p < 0.05$).

Serum concentrations of AFP. Concentrations of AFP were greater in autoimmune NZB dams treated with testosterone ($p < 0.05$ vs. NZB controls) on Day 18 of gestation, and an even greater elevation of AFP occurred in flutamide-treated NZB dams ($p < 0.01$ vs. NZB controls). A different pattern of AFP stimulation was observed in C57BL/6 mice. In this strain, concentration of AFP was greater in dams treated with testosterone than in C57BL/6 controls ($p < 0.01$), but serum concentration of AFP was not affected by therapy with flutamide.

Confirmation of Altered Fetal Endocrine Milieu Due to Maternal Treatment

Anogenital spaces were determined in 135 pups from testosterone-treated dams, 110 pups from flutamide-treated dams, and 135 pups from sham-treated dams. Within each treatment group, anogenital spaces did not differ as a function of hybrid type for either male or female offspring. Therefore, the data from NZB/W and C57/DBA2 mice were pooled within sex and treatment. No differences in anogenital spaces were detected among mice from vehicle-injected or sham-implanted groups of the same sex. The data from mice from these groups were pooled within sex and referred to as control. In control NZB/W fetuses, there was no overlap between female and male fetuses. In female fetuses born to dams implanted with testosterone, mean anogenital space was greater than in female fetuses from flutamide-treated and control dams (1.24 mm ± 0.02 vs. 1.00 ± 0.02 and 0.99 ± 0.02 mm, respectively). Anogenital space

in male offspring born to testosterone-treated and control dams did not differ ($p > 0.10$; 1.65 ± 0.02 and 1.64 ± 0.2, respectively). In offspring of flutamide-treated dams, masculinization of perineal tissue was inhibited completely and mean anogenital space was 1.00 ± 0.02 for all offspring, male and female.

Androgenic Responses in Male Offspring of Treated and Control Dams

Weights of seminal vesicles from 12-wk-old male offspring of treated and control dams are depicted in Table 1. The relationship between body weight and seminal vesicle weight was low ($r^2 = 0.21; p < 0.1$). In male offspring of testosterone-treated dams, seminal vesicle weights were increased slightly ($p = 0.09$) relative to controls. Flutamide treatment of the dam in late gestation resulted in small seminal vesicles; in 3 male offspring of these dams, seminal vesicles could not be identified and were analyzed as zero. Seminal vesicle weights of males from flutamide-treated NZB and C57BL/6 dams weighed less compared to seminal vesicle weights of males from control dams of the same strain (in each instance, $p < 0.001$). The mean of all seminal vesicle weights in NZB/W males was smaller than the mean of all seminal vesicle weights in C57/DBA2 males ($p < 0.001$).

Ovarian Function in Offspring of Treated and Control Dams

Within 36 hours of surgery, all ovarian transplants produced large preovulatory follicles, a response probably due to stress. One day later, corpora lutea formed and subsequently regressed. Identical progression was observed in NZB/W and C57/DBA2 female offspring of testosterone-treated dams and sham-treated control dams. Three of 6 NZB/W and 3 of 4 C57/DBA2 female offspring of dams implanted with testosterone displayed a second preovulatory follicle with subsequent formation of a corpus luteum. This progression was also observed in 3 NZB/W and C57/DBA2 females from sham-treated dams. The ovarian tissue of one NZB/W mouse from a testosterone-treated dam appeared to be healthy but inactive. Four days after surgery, transplanted ovarian tissue appeared to be necrotic in 3 females from testosterone-treated dams and in one female from a sham-treated dam.

The longevity study described below provided additional evidence that prenatal exposure to testosterone and hysterectomy did not alter ovarian morphology in NZB/W females. Ovaries from testosterone-exposed offspring resembled ovaries of intact NZB/W females from sham-treated dams. These ovaries were grossly and histologically normal and contained follicles in various stages of development and luteal tissue. The presence of luteal tissue in ovaries from these females suggested that ovulation was not inhibited by prenatal testosterone treatment.

Longevity in NZB/W Offspring of Testosterone-treated and Flutamide-Treated Dams

Twelve mice were excluded from analyses of longevity. Five mice that died with infections were excluded because these deaths were judged to be premature. In 7 instances, mice appeared to be ill, but complete necropsies and serological evaluations did not reveal the cause of death. Initially, life-spans in offspring of control NZB dams were analyzed. Mean age at death did not differ between NZB/W offspring of the same sex from sham-implanted and sham-injected dams. Data from these mice were, therefore, pooled within sex. In mice born to control dams, mean longevity was (±SEM) 33 ± 1 wk in female offspring (n = 34) and 48 ± 2 wk in male offspring (n = 29); this difference was significant ($p < 0.001$). This observation was in accord with the recognized predilection of NZB/W females for early-onset autoimmune disease and premature death compared to NZB/W males [16].

Figure 2 illustrates percentages of female NZB/W offspring surviving at 5-wk intervals during the longevity study. Mean ages at death in female offspring of testosterone-treated dams (34 ± 1 wk) and flutamide-treated dams (37 ± 2 wk) did not differ from controls. However, an apparent difference was noted between 30 and 40 wk of age, when numbers of surviving females from flutamide-treated dams exceeded numbers of living females from control dams. The median test [31] was employed to test whether these NZB/W females and female controls differed in central tendencies. Flutamide treatment of pregnant dams increased median longevity in female offspring to 35 wk, a significant increase compared to the median of 32 wk in corresponding female controls (chi square = 4.7; $p < 0.05$).

Figure 3 documents increased longevity in male NZB/W offspring of treated dams compared to controls. In males produced by dams treated with testosterone, prolongation of mean longevity to 61 ± 4 wk was greater than the mean life span of 48 ± 2 wk in males born to control females ($p < 0.001$). Flutamide treatment of NZB dams late in gestation also resulted in male offspring whose mean age at death (56 ± 3 wk) was delayed compared to the corresponding control group ($p < 0.05$).

TABLE 1. Mean seminal vesicle weights in 12-wk-old male offspring from testosterone-treated, flutamide-treated, and sham-treated (control) dams.[a]

Treatment of dams	Weight of seminal vesicles (gm)[b]			
	n	NZB/W	*n*	C57/DBA2
Testosterone	5	0.28 ± 0.02	11	0.34 ± 0.02
Flutamide	7	0.12 ± 0.02[c]	9	0.25 ± 0.03[c]
Control	12	0.24 ± 0.02	14	0.30 ± 0.02

[a]Male offspring, 16 wk of age, were bled at 1300 h and killed. Both seminal vesicles from each mouse were weighed.
[b]Mean ± SEM.
[c]Compared to control male offspring of corresponding sham-treated dams, $p < 0.001$.

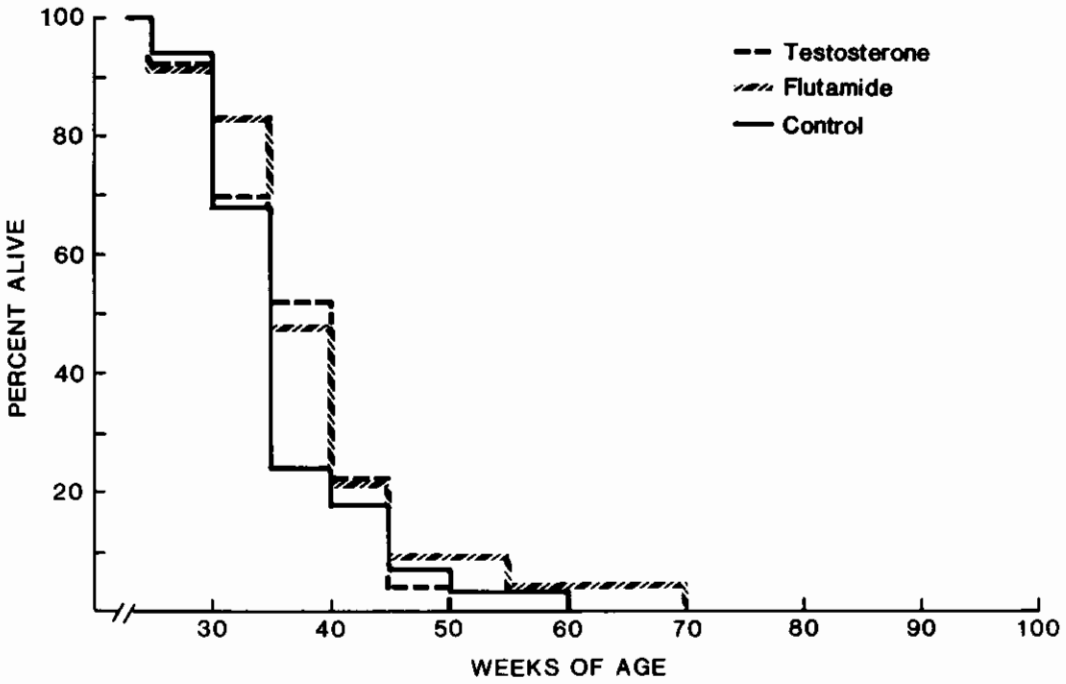

FIG. 2. Mortality curve for female NZB/W offspring from NZB dams treated from Days 13–18 of pregnancy, depicting 24 offspring of testosterone-treated dams, 23 offspring of flutamide-treated dams, 15 offspring of control dams receiving empty silastic capsules, and 19 offspring of control dams injected with vehicle. In female offspring of flutamide-treated dams, median age at death was delayed ($p < 0.05$ vs. controls).

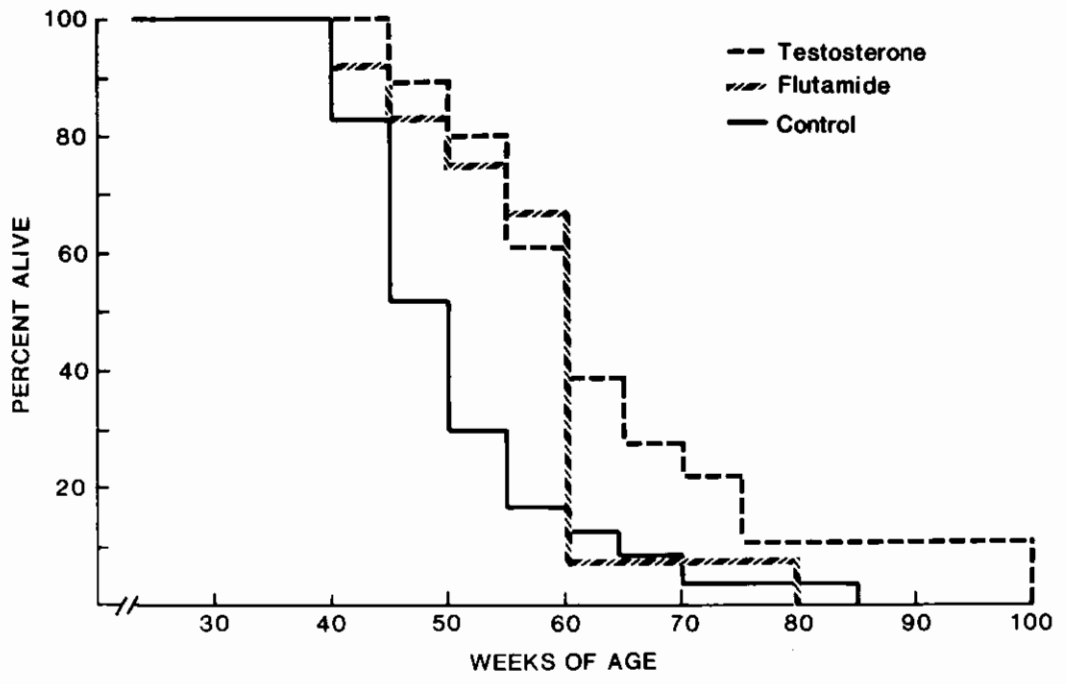

FIG. 3. Mortality in male NZB/W littermates of the mice illustrated in Figure 2. The males were offspring of NZB dams treated with testosterone (22 males) or flutamide (14 males). In the control group, dams implanted with empty capsules produced 14 males and vehicle-injected dams produced 15 male offspring. Mean life spans were prolonged significantly in male offspring of dams treated with testosterone ($p < 0.001$ vs. controls) or with flutamide ($p < 0.05$ vs. controls).

Causes of Death

Table 2 lists causes of death in NZB/W offspring of treated and control dams. Tissue was available for detailed histological examination in 96 percent of the mice entered into the longevity study. Based upon experience in this laboratory [26, 33] and the work of others [16], it was anticipated that autoimmune renal disease and vasculitis would cause death in the majority of NZB/W mice of both sexes. This supposition was confirmed in all groups of offspring. Treatment of NZB dams in late gestation with testosterone or flutamide, therefore, did not affect the frequency of immune-mediated disease in the NZB/W offspring. The appearance of malignant neoplasms in 3 percent of offspring was consistent with reports of spontaneous neoplasms occurring in 6–10 percent of untreated NZB/W hybrids [33; L.W. Keisler, unpublished observation]. The occurrence of neoplasms was not influenced by treatment of the dam. Infectious deaths were largely the result of fighting, with subsequent scrotal abscesses in males, and scattered cases of pneumonia and otitis media

DISCUSSION

The current study addressed the question that altering the maternal hormonal environment in late gestation might affect longevity in offspring with autoimmune disease (NZB/W hybrid mice). Female offspring of testosterone-treated NZB and C57BL/6 dams responded with masculinization of the external genitalia and vagina, but the dose of testosterone used in these experiments did not suppress the capacity to ovulate. Flutamide treatment of dams of both strains produced male offspring with reduced anogenital spaces and diminished seminal vesicle weights. Taken together, these morphological findings provide evidence that maternal treatment with testosterone or flutamide alters the hormonal environment of the developing fetuses.

Androgens have been reported to influence thymic morphology in rats [34] and change populations of lymphocytes in nonautoimmune mice [35]. Androgenic hormones exert widespread influences on cell-mediated immunity in autoimmune NZB/W mice, sustaining IL-2 production [36] and increasing T cell activity in castrated males [37]. These effects may contribute to the therapeutic effectiveness of testosterone in NZB/W hybrids [38]. It was expected, therefore, that exposure to exogenous testosterone during gestation could have a favorable influence on disease in NZB/W offspring. The longevity study provided evidence that responsiveness to prenatal testosterone occurred, but long-term effects were determined by the sex of the fetus: NZB/W males exposed in utero to exogenous testosterone had prolonged life spans, while females from the same litters showed no change in expected longevity. The basis for the increased susceptibility of male fetuses to the effects of testosterone remains to be defined. A sex-determined difference in the ability of adult NZB/W mice to generate antigen-specific suppressor cells has been identified [39], and reports from this laboratory have described sex-influenced responses to T-cell mitogens in NZB/W mice [27].

Interrelationships between the aberrant immune systems of New Zealand mice and the hormonal environments in which they develop appear to contribute to sex-influenced expression of disease as well as other aspects of physiology in this autoimmune model. For example, preliminary studies in this laboratory have provided data to support the hypothesis that the hypothalamic-pituitary-gonadal axis in NZB/W males differs from that in nonautoimmune C57/DBA2 males. Animals were bled at 12 wk of age and serum concentrations of testosterone were tested. In accord with the report of Coquelin and Desjardins [40], a testosterone pulse was defined as a concentration greater than 3.0 ng/ml. Numbers of adult NZB/W males exhibiting

TABLE 2. Deaths from renal disease/vasculitis (RD/V) and neoplasms (Neo) in autoimmune NZB/W offspring of testosterone-treated, flutamide-treated, and sham-treated (control) NZB dams.[a]

Treatment of dams	NZB/W female offspring				NZB/W male offspring			
	n	RD/V[b]	Neo	Other[c]	*n*	RD/V	Neo	Other[d]
Testosterone	24	21	0	3	22	16	2	4
Flutamide	23	21	1	1	14	12	0	2
Control	34	30	2	2	29	23	0	6

[a]NZB/W offspring of treated and control NZB dams were followed in a longevity study and killed when they developed signs of renal failure resulting from immune-mediated glomerulonephritis or evidence of neoplasms.

[b]RD/V (renal disease/vasculitis) = the spontaneous autoimmune disease of NZB/W mice caused death. These animals characteristically had proliferative glomerulonephritis (renal lesion score ≥ 35) and/or necrotizing vasculitis. Neo = cause of death was malignant neoplasm.

[c]In 5 instances, NZB/W females were found dead and tissue was lost by autolysis. One female was killed when she appeared to be moribund, but cause of death could not be established by necropsy.

[d]In 5 NZB/W males, infections (scrotal abscess, pneumonia, otitis media) caused death. Six males appeared to be moribund but the cause of death could not be established at necropsy. One male was found dead and was autolyzed.

testosterone pulses were diminished compared to the numbers of C57/DBA2 males exhibiting testosterone pulses (L.W. Keisler and S.E. Walker, unpublished observation].

Several factors may explain the beneficial effects of prenatal exposure to testosterone and flutamide on longevity. It may be postulated that flutamide acts in a manner similar to other hormone antagonists and binds to receptors for other sex hormones. It could also be that flutamide is an androgen blocker that acts as an agonist. If agonistic properties are present, they have not been detected in conventional assays of androgen activity [41, 42]. It is possible, however, that while flutamide is not agonistic in the reproductive system, it may act as an agonist in the immune system.

Flutamide may act in the fetus as it acts in adult mice, disrupting pituitary feedback and elevating serum concentrations of testosterone [32]. High concentrations of fetal androgen would be potentially beneficial. Flutamide, however, blocks androgen receptors [41] and the increased amounts of testosterone theoretically would be unable to bind to receptors and initiate changes in responsive cells in the immune system. In this situation, it could be argued that testosterone had indirect access to thymocytes through mechanisms that do not rely on sex steroid receptors. For example, testosterone alters secretion of prostaglandins in thymic epithelium [43]. If this mechanism is operational late in gestation, thymus-directed processing of T cells could be altered through mechanisms mediated by receptors for prostaglandins.

Serum concentrations of testosterone, estradiol, and AFP were assayed in NZB and C57BL/6 dams. Concentrations of estradiol in serum differed by strain. Serum concentrations of estradiol in pregnant NZB females were near the reported range for nonautoimmune mice on Day 18 of pregnancy [11, 12, 22], and values in treated and control NZB dams were not altered by treatment with testosterone or flutamide. Serum estradiol was consistently greater in C57BL/6 dams, and the heightened estradiol response in flutamide-treated C57BL/6 dams, compared to consistently normal levels in NZB dams, was unexpected. Serum concentrations of testosterone, a substrate for estradiol, were not different between NZB and C57BL/6 dams. One possible explanation is that aromatase, which converts testosterone to estradiol, may have been induced by flutamide in NZB dams [44].

Unlike testosterone, which does not have a specific serum-binding protein in the mouse [45], circulating estradiol is inactivated by binding to plasma AFP [9, 46]. AFP is considered an embryonic protein, and the majority of AFP detected in the pregnant mother is from the fetus [47]. Results of the current study support the hypothesis that high concentrations of exogenous testosterone stimulate an increase in serum AFP concentrations in both C57BL/6 and NZB pregnant mice, possibly as a result of an increase in secretion of AFP in fetuses.

What was surprising was the absence of a correlation between changes in AFP and total circulating estradiol in dams from the different treatment groups. The finding that treatment with testosterone led to elevated serum testosterone and AFP, but not estradiol, suggests that although the AFP which was induced by this treatment was immunoreactive with the antisera used in the RIA, the AFP may not have had the capacity for binding estrogen [48]. One would predict that a 3-fold increase in the concentration of a serum-binding globulin (such as AFP) would be reflected in an increase in the total serum concentration of ligand (estradiol), since the assay used for estradiol measured both unbound estradiol and estradiol bound to AFP. Flutamide treatment elevated total serum estradiol but not AFP in C57BL/6 dams, while an increase in serum AFP but not total estradiol was observed in NZB dams (the opposite response). There is simply no current explanation for these findings, but there is evidence that changes in circulating estradiol during fetal life can influence the differentiation of estrogen-sensitive tissues in rats and mice [8].

Elevated AFP levels in dams treated with testosterone or flutamide could explain increases in longevity observed in their male offspring. AFP may directly suppress immune reactions [49]. In the developing fetus, AFP may function as an immunosuppressive protein by binding and inactivating estrogens, thereby abrogating the immune-enhancing effects of these steroids. AFP may also have altered immune function in fetuses by binding circulating fatty acids and transporting them into tissues; in certain instances, this transport could be beneficial. For example, arachidonic acid, a precursor of prostaglandin E_1, has been shown to immunosuppress NZB/W mice and increase their longevity [50].

Recent studies [51, 52] have provided evidence that AFP regulates immune responses by affecting natural killer cell activity and expression of Ia determinants on macrophages. These cells make specific contributions to T-cell development in the fetal thymus [53–55]. Van Ewijk et al. [56] established that Ia antigens were located on the epithelium of the thymic rudiment, and were responsible for attracting blast cells involved in T-cell ontogeny in the developing thymus [57, 58]. AFP may therefore affect the immune system by regulating blast cell migration during fetal development. While our prenatal treatments were discontinued at birth, both testosterone and flutamide induced alterations in the reproductive and immune systems of NZB/W mice that were manifest later in life. High levels of endogenous AFP may have modulated development of immunologically active cells and resulted in NZB/W adults with retarded progression of autoimmune disease.

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