Male offspring exposed in utero to antiandrogens often display alterations in androgen-dependent developmental markers (e.g., anogenital distance [AGD], nipple retention) together with clearly adverse responses such as genital malformations and reproductive tract lesions. The objectives of this study were to determine whether in utero exposure to flutamide results in permanent changes in male AGD and nipple retention, characterize the dose-response relationship between flutamide-mediated alterations in these landmarks and clearly adverse antiandrogenic effects, and establish the predictive value and relationship between AGD and nipple retention, and other adverse manifestations. Male offspring were exposed in utero to 0, 6.25, 12.5, 25, or 50 mg/kg/day (po) of flutamide from gestation days 12 to 21. Offspring were uniquely identified at birth, and various androgen-mediated end points (AGD, areola/nipple retention, cryptorchidism, reproductive tract weights, and malformation incidence) were examined throughout life. In utero flutamide exposure significantly decreased the AGD on postnatal day (PND) 1 and increased areola/nipple retention in male rats on PND 13. Flutamide-induced alterations in AGD and areola/nipples in early postnatal life correlated with a reduction in AGD and retained nipples observed in the adult. Prenatal flutamide exposure resulted in dose-responsive increases in cryptorchidism. Hypospadias were observed in all flutamide-exposed offspring. In utero flutamide exposure induced partial or complete prostate agenesis and decreased the weights of the seminal vesicles, levator ani bulbocavernosus (LABC) muscle, testes, and epididymides in a dose-dependent manner. Epididymal malformations were observed mainly in the 50 mg/kg/day flutamide dose group. In general, flutamide-induced alterations in dihydrotestosterone (DHT)- and testosterone (T)-dependent development each had similar respective dose-response curves. DHT-mediated development was more sensitive to in utero flutamide exposure than T-dependent processes. However, the dose-response curves for flutamide-induced changes in cryptorchidism and seminal vesicle weight were intermediate between the dose-response curves for DHT- and T-mediated development, indicating that proper development of these tissues may require both androgens. The LABC also displayed a dose-dependent decrease in weight that was similar to dose-response observed with seminal vesicle weight and was the most sensitive T-dependent end point measured. Flutamide-induced decreases in AGD predicted subsequent malformations as evidenced by logistic regression and receiver operator characteristic analysis of malformations versus AGD. However, the AGD that would predict a 10% incidence of seminal vesicle malformations is equivalent to a female AGD. An almost fully feminized phenotype of 10–12 nipples was observed in animals that had malformations in T-dependent tissues, whereas 6 or more nipples were observed in animals with malformation in DHT-dependent tissues. These data suggest that flutamide-mediated changes in AGD and nipple retention are not sensitive predictors of altered T-mediated development.

Key Words: flutamide; anogenital distance; nipple retention; dose-response; malformations; antiandrogen; reproductive development.

Exposure to environmental contaminants with endocrine-like activity has been hypothesized to cause a number of reproductive and other deficits in humans and wildlife (Gray et al., 1998; Sharpe and Skakkebaek, 1993; Toppari et al., 1996). In addition to their potential for inducing estrogenic effects, recent evidence indicates that certain environmental compounds also alter androgen-dependent reproductive development in rodents (Cook et al., 1993; Gray and Kelce, 1996; Gray et al., 1994; Kelce et al., 1998, 1997; LeBlanc et al., 1997; McIntyre et al., 2000; Mylchreest et al., 1998, 1999). Moreover, infants and young children may be more sensitive to endocrine-active compounds (Foster, 1998). These concerns have been reflected in the Safe Drinking Water Act and Food Quality Protection Act, which require the United States Environmental Protection Agency (U.S. EPA) to develop methodologies that identify compounds with potential endocrine activity.

In the rat and human, fetal androgen production during gestation is required for normal male sexual differentiation (Schardein, 1993). Testosterone (T) is necessary for proper
development of the testes as well as differentiation of the Wolffian ducts into the epididymides, vasa deferentia, and seminal vesicles, whereas dihydrotestosterone (DHT), locally produced from T by 5α-reductase, stimulates normal differentiation and development of the genital tubercle and urogenital sinus into the prostate and external genitalia (Berman et al., 1995; Clark et al., 1993; Imperato-McGinley et al., 1992; Kassim et al., 1997; Roy and Chatterjee, 1995; Silversides et al., 1995; Veyssiere et al., 1982; Wilson and Lasnitski, 1971). In addition, the growth of the levator ani bulbocavernosus (LABC) muscle is also T-dependent (Blohm et al., 1986). The development of the rodent nipple is sexually dimorphic (Kratochwil, 1971; Kratochwil and Schwartz, 1976). Although mammary gland development begins similarly in both male and female rodents, female rats and mice have nipples while males do not. In the developing rodent fetus, DHT produced locally from fetal T causes regression of the nipple anlagen (Imperato-McGinley et al., 1986; Kratochwil, 1977, 1986). This process is blocked by fetal exposure to antiandrogens, and these offspring subsequently display nipples.

In the rat, exposure to antiandrogenic compounds during late gestation alters androgen-dependent reproductive development (Gray et al., 1994, 1999; McIntyre et al., 2000; Mylchreest et al., 1998, 1999; You et al., 1998). Antiandrogen-mediated decreases in anogenital distance (AGD, the distance from the sex papilla to the anus) and the retention of nipples has been associated with adversely affected development of androgen-dependent reproductive tissues (Clark et al., 1990; Gray et al., 1999; Hecker et al., 1980; Imperato-McGinley et al., 1986; McIntyre et al., 2000; Mylchreest et al., 1999; Ostby et al., 1999; You et al., 1998). Pre- and postnatal exposure to antiandrogens has also been shown to decrease the weight of the LABC in the male rat (Gray et al., 1999; Lambright et al., 2000; Mylchreest et al., 2000; Ostby et al., 1999; U.S. EPA, 1998a).

The correlation between antiandrogen-mediated alterations (e.g., decreased AGD and nipple retention) and adverse and irreversible malformations in androgen-dependent development is unclear. Moreover, confusion exists about the biological significance of both the antiandrogen-induced decreases in AGD and retention of areolae/nipples observed in early postnatal male rats. Studies examining the effects of in utero exposure to finasteride, a 5α-reductase inhibitor, demonstrated that finasteride exposure decreases AGD at birth. However, these male offspring displayed “catch up growth” and adult animals exposed to low doses of finasteride displayed AGDs similar to control animals, suggesting that decreases in AGD seen in early postnatal life are transient (Clark et al., 1993, 1990). These investigators also observed that areolae/nipples observed in early postnatal rats are temporary (Clark et al., 1990). In contrast to these previous studies with finasteride, rats exposed to the antiandrogens di(4-nitro-3-trifluoromethyl isobutyl)phthalate, diethylhexylphthalate, and linuron displayed retained nipples at both PND 13 and the adult (PND 180–270) necropsy (Gray et al., 1999).

The U.S. EPA endocrine disruptor screening program is divided into screening (Tier 1) and testing (Tier 2). The goal of Tier 1 is to determine whether a chemical interacts with estrogen, androgen, or thyroid systems, whereas the purpose of Tier 2 is to determine whether a chemical substance, potentially identified in Tier 1, exhibits endocrine-mediated adverse effects in a model animal system and to identify, characterize, and quantify those effects (U.S. EPA, 1998a). AGD is included in the OPPTS Effects Test Guidelines for Reproduction and Fertility Effects, the suggested definitive Tier 2 test for assessing endocrine-mediated effects (U.S. EPA, 1998a). The AGD is measured on the day of birth for all F2 pups if there is a treatment-related effect detected in F1 sex ratio or sexual maturation (U.S. EPA, 1998b). Moreover, it has been recommended that additional end points of nipple retention, preputial separation, and the weight of the LABC be included in this test. The weight of the LABC, in addition to other reproductive organ weights, is included in both the EDSTAC rodent 20-day prepubertal and Hershberger (Tier 1) assays (U.S. EPA, 1998a). However, the respective sensitivity of these androgen-dependent end points in relation to other androgen-dependent indicators is unknown.

Flutamide (4′-nitro-3′-trifluoromethyl-isobutyluridine) is a potent nonsteroidal androgen receptor antagonist that has been used therapeutically to treat androgen-dependent prostate cancer (Delaere and Van Thillo, 1991; Murphy et al., 1991) and as a tool to study male reproductive development. Studies in rats have demonstrated that pre- or postnatal flutamide exposure alters androgen-dependent reproductive development and function (Imperato-McGinley et al., 1992; Kassim et al., 1997). In a limited dose-response study, male rat pups from pregnant dams injected sc with various flutamide concentrations (18–300 mg/kg/day) from gestation days 12 through 21 displayed decreased reproductive organ weights, feminization of male external genitalia, altered androgen-dependent testicular descent, and retention of nipples (Imperato-McGinley et al., 1992). In a definitive Tier 2 study, male rat pups from pregnant dams injected sc with 1, 3, and 10 mg/kg/day of flutamide from gestation days 12 through 21 displayed decreased reproductive organ weights, feminization of male external genitalia, altered androgen-dependent testicular descent, and retention of nipples (Imperato-McGinley et al., 1992). In a definitive Tier 2 study, male rat pups from pregnant dams injected sc with 1, 3, and 10 mg/kg/day of flutamide from gestation days 12 through 21 displayed decreased reproductive organ weights, feminization of male external genitalia, altered androgen-dependent testicular descent, and retention of nipples (Imperato-McGinley et al., 1992).
mine model significance) and subsequent receiver operator characteristic function (predictive ability) were used to determine whether flutamide-mediated decreases in AGD on postnatal day (PND) 1 predicted malformation of androgen-dependent tissues in adult animals.

MATERIALS AND METHODS

Animals. This study was conducted in accordance with federal guidelines for the care and use of laboratory animals (National Academy of Sciences, National Research Council, 1996) and was approved by the Institutional Animal Care and Use Committee at the CIIT Centers for Health Research (CIIT). Animals were housed in the CIIT animal care unit, a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Animals were kept in a HEPA-filtered, mass air-displacement room with a 12-h light-dark cycle at 18–26°C and relative humidity of 30–70%. Animals had access ad libitum to deionized water and rodent chow (NIH-07, Zeigler Brothers, Gardners, PA). Time-mated, 8–10-week-old, nulliparous CRL:CD (SD)Br rats were obtained from Charles River Laboratories Inc. (Raleigh, NC) on gestation day (GD) 0. GD 0 was defined as the day that sperm was found in the vagina of the mated female. Animal allocation to treatment groups was done by body weight randomization to ensure unbiased weight distribution among groups. Individual dams and offspring were housed in polycarbonate cages on ALPHA-dri bedding (Shepherd Specialty Papers, Kalamazoo, MI) until weaning (PND 21), at which time animals were group-housed, up to 4 per cage, by sex and treatment. After PND 60, all male littermates were transferred, 2–3 per cage, to stainless steel-wire cages until necropsy. Female offspring were euthanized by CO2 asphyxiation on PND 60 and were not subjected to detailed postmortem examination.

Treatment. Sperm-positive animals, 11–12 dams per dose for dose levels of 0–25 mg/kg/day and 7 dams for the 50 mg/kg/day dose level, were gavaged daily (0800–1030 h) from GD 12 to 21 with either corn oil (Sigma) (2 mL/kg/day) or flutamide at 6.25, 12.5, 25, or 50 mg/kg/day (2 mL/kg/day). These dose levels were chosen based on previous studies that demonstrated sc flutamide injections (from GD 12 to 21) of 18 mg/kg/day decreased seminal vesicle weights (Imperato-McGinley et al., 1992). Dams were examined daily for clinical signs of toxicity. Dam body weights were recorded daily during dosing and weekly during lactation.

Androgen-dependent reproductive end points. On the day of delivery, which was considered to be PND 1, pups were counted and examined for signs of clinical toxicity. Pups were uniquely identified by foot tattoo, and the AGD was measured using a dissecting microscope with an eyepiece reticle (accuracy 0.05 mm). The AGD for all pups was measured by an individual investigator who was unaware of animal exposure levels. Definitive sex of offspring was determined after weaning. Pup litter weights (by sex and litter) and individual pup body weights after weaning were collected weekly. On PND 13, male pups were inspected by a single investigator who was unaware of animal exposure levels for the presence and number of areolae, nipples, or both. No distinction was made between the retention of an areola or nipple on PND 13 and therefore referred to as areolae/nipples. Beginning on PND 38, male pups were examined daily for malformations of the external genitalia, testicular descent, and preputial separation.

Necropsy of dams. Pups were weaned on PND 21, and dams were euthanized by CO2 asphyxiation. Body and organ weights (liver, kidneys, adrenals, uterus, and ovaries) and number of implantation sites were recorded.

Necropsy of F1 animals. Sexually mature (PND 95–105) male rats were euthanized by decapitation, and trunk blood was collected. Following blood collection, the ventral surface of the animal was shaved for counting the number of nipples and the AGD measured with a dial caliper. The external genitalia, including the scrotum, prepuce, and penis, were visually inspected. Gross internal examination of the reproductive tract included inspection of the testes, epididymides, vasa deferentia, prostate, bulbourethral glands, seminal vesicles, and coagulating glands. Additionally, the liver, kidneys, and adrenal glands were grossly examined. Body and organ weights (epididymides, vasa deferentia, testes, ventral prostate, dorsolateral prostate, seminal vesicles and coagulating glands [with fluid], liver, kidneys, adrenals, and LABC muscle) were collected. Tissues were fixed in either Bouin’s fixative (right testes and epididymides) or 10% neutral buffered formalin, processed, paraffin-embedded, sectioned (5 μm), and stained with hematoxylin and eosin. The left testes and epididymides were retained for ancillary studies.

Dose-response curves. For AGD and areola/nipple retention, a female AGD (1.20 mm, determined from untreated controls) and 12 nipples/rat were considered maximal (100%) responses on PND 1. Changes in AGD, areola/nipple retention, and organ weights are represented as percent difference (absolute) from control. Unilateral and bilateral cryptorchid testes and malformation responses are presented as individual and litter incidences. Curves were generated by Sigma Plot (version 5.0, SPSS, Inc., Richmond, CA).

Statistical analysis. Statistical analyses were conducted using JMP (version 4.0.0, SAS Institute, Cary, NC). Normality (Shapiro-Wilk) and homogeneity (Bartlett) assumptions were tested prior to analysis. Pup data was analyzed both individually and nested by dam to yield litter means. Either ANOVA or ANOVA was used to test for significance of treatment effects, and the covariates are defined in figure legends. If the p-value for treatment effects was less than 0.05, post hoc comparisons of either Dunnett’s (for ANOVA) or contrasts of least square means were used to assess the significance of treatment differences. The Bonferroni correction was applied for post hoc ANCOVA analysis. The correlation between the AGD on PND 1 and PND 100 was determined by linear regression. Since the number of nipples per rat was not normally distributed and is a noncontinuous variable, the relationship between retained nipples on PND 13 and PND 100 was determined by contingency analysis followed by the Cochran-Mantel-Haenszel test. Logistic regression was used to determine if flutamide-mediated decreases in AGD on PND 1 were associated with malformation of androgen-dependent tissues in adult animals and was considered significant if p < 0.05. After logistic regression, the receiver operator characteristic (ROC) and inverse prediction functions were employed. ROC is a graphic display that gives a measure of the predictive accuracy of the logistic regression model and presented as area under the curve (AUC). AUC values approaching 1 are fully predictive, whereas values approaching 0.5 are not predictive (Hanley and McNeil, 1982). The inverse prediction function of the logistic regression analysis was used to determine the AGD (and the respective fiducial confidence intervals) at which 10 and 50% of the pups would display a given malformation.

RESULTS

Effects of Flutamide Exposure on Pregnancy and Reproductive Performance

Dams treated with 50 mg/kg/day of flutamide from GD 12 to 21 displayed a small (8%) but significant decrease in body weight on GD 21 when compared to vehicle control treated animals. Body weight of dams in the other dose groups was not significantly altered. Body weight gain during treatment was significantly decreased in the 25 and 50 mg/kg/day dose group by 12 and 18%, respectively. All dams were pregnant and littered normally. However, 1 dam in the 12.5 mg/kg/day dose group cannibalized her offspring within 24 h. Dam body weight on PND 7 was significantly decreased by approximately 10% in both 25 and 50 mg/kg/day dose groups. By PND 14 dam body weight in flutamide-treated dams were similar to control dams (data not shown). Least square means (terminal body weight as a covariate) for dam organ weights (kidney,
liver, uterus, ovaries, and adrenals) were comparable to control dams (data not shown). The number of implantation sites and the number of live pups born per litter were similar and not significantly altered by flutamide exposure, indicating that immediate cannibalization of neonates was limited to the one litter in the 12.5 mg/kg/day exposure group (data not shown).

The proportion of pups born alive and surviving to weaning was not significantly affected by flutamide exposure, although pup survival was slightly decreased in the 12.5 mg/kg/day dose group as a result of the 1 litter in the 12.5 mg/kg/day dose group being cannibalized. In utero flutamide exposure did not alter sex ratio or male pup weight at PND 1, 21, or 100 (data not shown). Flutamide-exposure did not alter either the AGD or the pup weights of female offspring (data not shown).

**Effects of Flutamide-Exposure on Postnatal End Points**

*In utero* flutamide exposure significantly decreased the AGD of male offspring on PND 1 in a dose-responsive manner. Male offspring displayed decreases of 43, 49, 49, and 53% in the 6.25, 12.5, 25, and 50 mg/kg/day dose groups, respectively (Fig. 1A). On PND 1, the AGD (adjusted for body weight by covariate analysis) in control and exposed male pups displayed a normal distribution (Fig. 1B). The mean AGD for control female pups was 1.20 mm with a range of 1.05 to 1.30 mm (Fig. 1B). The AGD measured at necropsy on PND 100 displayed a trend similar to that observed on PND 1 (Fig. 1A). The adjusted AGD of both control and exposed males displayed a normal distribution (Fig. 1C). The coefficient of determination ($r^2$) for the linear regression of AGD on PND 1 versus AGD on PND 100 was 0.73 (Fig. 1A, inset).

On PND 13, the litter mean for number of retained areolae/nipples per pup from vehicle-control dams was less than 1 areola/nipple per pup. Late gestational flutamide exposure resulted in a dose-responsive increase in the litter mean number of areolae/nipples retained per pup. These animals displayed 10.2, 11.5, 12, and 12 areolae/nipples per rat in the 6.25, 12.5, 25, and 50 mg/kg/day dose groups (Fig. 2A). On PND 13, areolae/nipples were observed in about 12% of the control animals, and 3 of these pups exhibited more than 5 areolae/nipples (Fig. 2B). In utero flutamide exposure resulted in 100% of the animals displaying areolae/nipples. In the 6.25 mg/kg/day dose group, approximately 80% of the pups exhibited 10 or more areolae/nipples (Fig. 2). At dose levels of 12.5 mg/kg/day, approximately 95% of the pups displayed 12 areolae/nipples with the remaining pups having 10–11 areolae/nipples per male pup. At the 25 and 50 mg/kg/day dose levels, male pups displayed a fully feminized areolae phenotype with 12 areolae/nipples per male pup (Fig. 2B).

Adult male offspring were inspected for the retention of nipples on PND 100. Male rats exposed to vehicle control displayed a low incidence of retained nipples and the total litter mean was 0.1 nipple/rat. Two (out of 76 total) adult animals in the control group displayed 1 and 4 nipples, respectively.

Areolae/nipples were not observed in either of these animals on PND 13. Flutamide exposure increased the mean number of retained nipples per adult male rat. These animals exhibited

![FIG. 1. Effect of prenatal flutamide exposure from gestation day 12 to 21 on anogenital distance (AGD). Values are nested litter means, body weight used as a covariate, and expressed as the mean number of areolae and/or nipples per rat (A). * = Significantly different from control (p < 0.05). Linear regression of AGD on PND 1 vs. PND 100 (Inset). Coefficient of determination ($r^2$) was 0.73. AGD was adjusted for body weight by covariate analysis prior to linear regression. Distribution of AGDs in control and flutamide-exposed male rats on PND 1 (B) and 100 (C). The respective AGDs from these offspring exhibited a normal distribution both collectively across and within the flutamide dose levels.](https://academic.oup.com/toxsci/article-abstract/62/2/236/1663633)
8.25, 10.1, 11.5, and 12 nipples/rat, respectively (Fig. 2A). In the 6.25 mg/kg/day dose group, approximately 45% of flutamide-exposed adult male animals exhibited 10 or more nipples, whereas 55% of the animals displayed 6–9 nipples (Fig. 2B). At the 12.5 mg/kg/day dose level, 65% of the male rats displayed the fully feminized phenotype of 12 nipples with 30% displaying 10 or 11 nipples. Approximately 85 and 95% of the male rats displayed fully feminized phenotype of 12 nipples in the 25 and 50 mg/kg/day dose groups, respectively (Fig. 2B). Contingency analysis of the number of nipples on PND 13 versus PND 100 and subsequent Cochran-Mantel-Haenszel test (blocked for dose) indicated a linear association between these 2 end points.

Litter means for the onset of puberty, as determined by complete separation of the prepuce from the ventral surface of the glands penis, could not be determined since 100% of the flutamide-exposed animals exhibited hypospadias (Fig. 3). In these animals, there was a cleft in the ventral surface of the penis, and the os penis was often exposed. In addition to hypospadias, many of these animals also displayed vaginal pouches (Fig. 3). Upon necropsy, the \textit{vas deferens} of several of these animals could be traced to the opening of the vaginal pouch where sperm was detected. Testicular descent was significantly impaired by \textit{in utero} flutamide exposure with approximately 55 (11/12 litters), 71 (10/10 litters), 76 (11/11 litters), and 85% (7/7 litters) of the adult males displaying either unilateral or bilateral cryptorchidism in the 6.25, 12.5, 25, and 50 mg/kg/day dose groups, respectively (Fig. 4). Cryptorchid testes most often descended through the inguinal canal and were located ectopically in the subcutis of the inguinal area enclosed by an extension of the peritoneal cavity. When probed, a pathway of descent to the scrotum could not be identified.

![FIG. 2. Effect of prenatal flutamide exposure from gestation day 12 to 21 on areola/nipple retention on postnatal day (PND) 13 and 100 (A). Distribution of areolae/nipples in individual male offspring on PND 13 and 100 in control and flutamide-exposed rats (B). No distinction was made between an areola and a nipple on PND 13, and only nipples identified grossly after shaving the animals were counted on PND 100.](https://academic.oup.com/toxsci/article-abstract/62/2/236/1663633)

![FIG. 3. Flutamide-exposed male displaying altered reproductive development. Animal was exposed to 25 mg/kg/day from gestation day 12 to 21. Note the vaginal pouch opening, abnormal penis with a cleft prepuce, retained nipple, and undescended testis.](https://academic.oup.com/toxsci/article-abstract/62/2/236/1663633)

![FIG. 4. Prenatal flutamide exposure blocks testicular descent in male offspring. Bar represents the percentage of pups displaying uni- or bilateral cryptorchid testes. The number of responding litters is noted above each bar.](https://academic.oup.com/toxsci/article-abstract/62/2/236/1663633)
Adverse Flutamide-Mediated Lesions Observed at Necropsy

At necropsy on PND 100, testicular hypoplasia was observed in flutamide-exposed offspring affecting 5% (2/11 litters), 24% (10/12 litters), 29% (8/10 litters), 49% (11/11 litters), and 93% (7/7 litters) of the right testes and 5% (3/11 litters), 43% (10/12 litters), 63% (10/10 litters), 57% (10/11 litters), and 80% (7/7 litters) of the left testes in the 0, 6.25, 12.5, 25, and 50 mg/kg/day dose groups, respectively (Table 1). These testes were small and had decreased weights when compared to control animals (Figs. 5A and 5B, Table 2). Both descended and cryptorchid testes displayed moderate to severe degeneration of the seminiferous epithelium that was characterized by variable thinning of the seminiferous epithelium due to decreased numbers (to complete absence) of spermatogonia, spermatocytes, and spermatids (Fig. 6, Table 3). In addition, cell debris and large multinucleated spermatids in the lumina were often present. Though difficult to assess histologically, there was apparent interstitial edema in many of the testes, especially at the higher exposure levels. Small numbers of testes were necrotic with severe neutrophilic and granulomatous orchitis. Grossly these testes were firm, tan, and had a rough capsular surface, which was often adhered to the musculature of either the scrotum or the muscle in the ectopic location. Occasional non-necrotic testes had small amounts of inflammation, most often centered on one or several individual tubules.

Flutamide exposure also resulted in a dose-dependent increase in epididymal lesions and included hypoplasia and/or absence of the head, body, and tail of the epididymis (Table 1, Figs. 5A and 5B). Epididymal malformations were most prevalent in the 50 mg/kg/day dose group with 73 and 56% of the

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**TABLE 1**

Incidence of Abnormal Androgen-Dependent Tissues in 100-Day-Old Rats Exposed to Flutamide *in Utero*

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Diagnoses</th>
<th>0 (6.25)</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right testis*</td>
<td>Abnormal*</td>
<td>4/76 (2/11)*</td>
<td>16/67 (10/12)</td>
<td>17/59 (8/10)</td>
<td>26/53 (11/11)</td>
</tr>
<tr>
<td>Right epididymis*</td>
<td>Malformed*</td>
<td>0/76 (0/11)</td>
<td>1/67 (1/12)</td>
<td>1/59 (1/10)</td>
<td>6/53 (4/11)</td>
</tr>
<tr>
<td>Left testis*</td>
<td>Abnormal*</td>
<td>4/76 (3/11)</td>
<td>29/67 (10/12)</td>
<td>37/59 (10/10)</td>
<td>30/53 (10/11)</td>
</tr>
<tr>
<td>Left epididymis*</td>
<td>Malformed*</td>
<td>0/76 (0/11)</td>
<td>2/67 (2/12)</td>
<td>0/59 (0/10)</td>
<td>3/53 (3/10)</td>
</tr>
<tr>
<td>Vasa deferentia*</td>
<td>Malformed*</td>
<td>0/76 (0/11)</td>
<td>1/67 (1/12)</td>
<td>2/59 (1/10)</td>
<td>4/53 (4/11)</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>Absence*</td>
<td>0/76 (0/11)</td>
<td>0/67 (0/12)</td>
<td>1/59 (1/10)</td>
<td>0/53 (0/11)</td>
</tr>
<tr>
<td>Ventral prostate</td>
<td>Absence*</td>
<td>0/76 (0/11)</td>
<td>25/67 (9/12)</td>
<td>52/59 (10/10)</td>
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<td>Dorsolateral prostate</td>
<td>Absence*</td>
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<td>18/67 (8/12)</td>
<td>48/59 (10/10)</td>
<td>51/53 (11/11)</td>
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<tr>
<td>Bulbourethral glands*</td>
<td>Absence*</td>
<td>0/69 (0/11)</td>
<td>42/59 (12/12)</td>
<td>47/51 (10/10)</td>
<td>47/47 (11/11)</td>
</tr>
</tbody>
</table>

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**FIG. 5.** Effects of prenatal flutamide exposure on androgen-dependent development in male offspring on postnatal day (PND) 100. Testes and epididymides on PND 100 from offspring of dams administered corn oil control (A) or 25 mg flutamide/kg/day (B). Note the decreased size of the flutamide-exposed testis and agenesis of the body and tail of the epididymis. Prostate and seminal vesicles on postnatal day 100 of dams administered corn oil control (C) or flutamide (D). Note the absence of the dorsolateral and ventral prostate lobes and abnormally shaped seminal vesicles. *In situ* photograph of *levator ani* bulbocavernosus muscles in male offspring on PND 100 exposed *in utero* to corn oil (E) or flutamide (F). Note the decrease in size of the LABC and absence of bulbourethral glands.

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*a* Includes scrotal and nonscrotal testes and epididymides.

*b* Hypoplastic, excludes testes with inflammatory lesions.

*c* Number adult rats responding/total number rats.

*d* Number of litters responding/total number of litters.

*e* Includes partial to complete agenesis of the epididymis and *vas deferens*, but complete, but small epididymides are not included.

*f* Tissue was not present.

*g* Unilateral or bilateral.
rats (7/7 of the litters) displaying this lesion on the right and left sides, respectively. Cryptorchid/ectopic testes and epididymides (if present) from flutamide-exposed animals were hypoplastic. Even when these testes are excluded from analysis, flutamide exposure induced epididymal hypoplasia, in addition to agenesis, in the 50 mg/kg/day dose level group (Tables 1 and 2). Epididymides from flutamide-exposed animals that were not grossly malformed (all portions present) had decreased diameter of ductules with thickening of the epithelium. This thickened epithelium was most prominent in the body and tail of the epididymides. In incomplete epididymides, there was a spectrum of lesions, including decreased numbers of ductules, thickened epithelium (seen mostly in the body and tail), cellular debris, and decreased numbers or absence of sperm (Fig. 6). Epididymitis was also present along with the orchitis (Table 3). Although most epididymides had focal or multifocal abscesses or granulomas, small numbers had diffuse inflammation throughout the organ. The infiltrates seen were predominantly neutrophilic and granulomatous. Similar to testes with orchitis, inflamed epididymides were occasionally adhered to the muscle of the scrotum, the muscle of the ectopic location, or to the testes themselves.

Prenatal flutamide exposure caused dose-dependent increases in unilateral and bilateral malformations of the *vasa deferentia* affecting 1.5 (1/12 of the litters), 3.4 (1/10), 7.5 (4/11 of the litters) and 65% (7/7 of the litters) of the animals in the 6.25, 12.5, 25, and 50 mg/kg/day dose groups, respectively (Table 1). In *utero* flutamide exposure induced seminal vesicle agenesis and was observed in 1.7 (1/10 litters) and 11% of the animals (2/7 of the litters) in the 12.5 and 50 mg/kg/day dose levels, respectively (Table 1). These lesions were not observed in the 6.25 and 25 mg/kg/day exposure groups.

Seminal vesicle weight was decreased in a dose-responsive manner (Table 2). Seminal vesicles in flutamide-exposed animals were small, abnormally shaped, lacked clear division between the 2 lobes, and did not have the typical curled shape. These tissues contained markedly less fluid than control animals and often no fluid could be expressed. Histologically, the epithelium appeared normal, but the organ was small and had less glandular surface area.

Prenatal flutamide exposure induced agenesis of the prostate with 37 (9/12 of the litters), 88 (10/10 of the litters), 96 (11/11 of the litters), and 98% (7/7 of the litters) of the male offspring displaying malformed ventral prostates and 27 (8/12 of litters),
81 (10/10 of the litters), 96 (11/11 of the litters), and 98% (7/7 of the litters) of the male offspring displaying agenesis of the dorsolateral prostate in the 6.25, 12.5, 25, and 50 mg/kg/day dose levels, respectively (Figs. 5C and 5D, Table 1). Grossly normal prostates in the 6.25 mg/kg/day dose group weighed 50% less than those of controls (Table 2). Microscopically, ducts were decreased in number, dilated and lined by flattened epithelium.

Approximately 70% of the rats (12/12 of the litters) in 6.25 mg/kg/day dose group displayed either unilateral or bilateral absence of the bulbourethral glands. At higher dose levels, these glands were absent in all of the male offspring (Figs. 5E and 5F, Table 1). Prenatal flutamide exposure decreased the weight of the LABC in a dose-dependent fashion (Figs. 5E and 5F, Table 2). The weights of the adrenal glands, kidneys, and liver were unaffected by prenatal flutamide exposure (data not shown).

### Dose-Response Relationships among Markers of Altered Androgen-Dependent Development and Other Adverse Manifestations

The dose-response curves for flutamide-induced changes in the DHT-dependent tissues (AGD, retention of areolae on PND 13, retention of nipples on PND 100, prostate malformations, bulbourethral gland agenesis, and hypospadias) were similar

#### TABLE 3

| Organ Weights of PND 100 Male Rats Exposed in Utero to Flutamide during Gestation Days 12 to 21 | Flutamide (mg/kg/day) |
| --- | --- | --- | --- | --- |
| 0 | 6.25 | 12.5 | 25 | 50 |
| Right testis*<sup>a</sup> | 1.79 ± 0.07 (11) | 1.60 ± 0.07 (12) | 1.52 ± 0.07 (10)* | 1.42 ± 0.07 (11)* | 1.00 ± 0.08 (7)* |
| Right testis (descended)*<sup>a</sup> | 1.79 ± 0.06 (11) | 1.80 ± 0.06 (12) | 1.78 ± 0.06 (10) | 1.73 ± 0.06 (11) | 1.14 ± 0.08 (6)* |
| Right testis (cryptorchid) | No tissue<sup>c</sup> | 0.93 ± 0.10 (8) | 0.81 ± 0.11 (7) | 1.01 ± 0.09 (11) | 0.82 ± 0.10 (7) |
| Right epididymis | 0.61 ± 0.02 (11) | 0.53 ± 0.02 (12)*<sup>b</sup> | 0.54 ± 0.02 (10)*<sup>b</sup> | 0.45 ± 0.02 (11)*<sup>b</sup> | 0.32 ± 0.05 (3)*<sup>b</sup> |
| Right epididymis (descended)<sup>b</sup> | 0.61 ± 0.02 (11) | 0.60 ± 0.02 (12) | 0.61 ± 0.02 (10) | 0.57 ± 0.02 (11) | 0.33 ± 0.05 (3)*<sup>b</sup> |
| Right epididymis (cryptorchid) | No tissue<sup>c</sup> | 0.29 ± 0.03 (10) | 0.32 ± 0.03 (10) | 0.29 ± 0.02 (11) | 0.33 ± 0.04 (3) |
| Left testis | 1.80 ± 0.08 (11) | 1.30 ± 0.08 (12)*<sup>b</sup> | 1.17 ± 0.09 (10)*<sup>b</sup> | 1.31 ± 0.09 (11)*<sup>b</sup> | 1.01 ± 0.11 (7)*<sup>b</sup> |
| Left testis (descended)<sup>b</sup> | 1.82 ± 0.07 (11) | 1.73 ± 0.09 (10) | 1.88 ± 0.11 (8) | 1.71 ± 0.10 (11) | 1.22 ± 0.13 (6)*<sup>b</sup> |
| Left testis (cryptorchid) | No tissue<sup>c</sup> | 0.82 ± 0.06 (8) | 0.87 ± 0.06 (10) | 0.96 ± 0.06 (11) | 0.90 ± 0.06 (7) |
| Left epididymis | 0.57 ± 0.03 (11) | 0.41 ± 0.03 (12)*<sup>b</sup> | 0.36 ± 0.03 (10)*<sup>b</sup> | 0.37 ± 0.03 (11)*<sup>b</sup> | 0.31 ± 0.04 (7)*<sup>b</sup> |
| Left epididymis (descended)<sup>b</sup> | 0.57 ± 0.01 (11) | 0.54 ± 0.02 (10) | 0.56 ± 0.02 (9) | 0.52 ± 0.02 (9) | 0.34 ± 0.03 (4)*<sup>b</sup> |
| Left epididymis (cryptorchid) | No tissue<sup>c</sup> | 0.26 ± 0.02 (8) | 0.27 ± 0.02 (10) | 0.27 ± 0.02 (11) | 0.26 ± 0.03 (7) |
| Vasa deferentia<sup>c</sup> | 0.197 ± 0.003 (11) | 0.180 ± 0.004 (12)*<sup>b</sup> | 0.178 ± 0.004 (10) | 0.184 ± 0.004 (11) | 0.164 ± 0.007 (7)*<sup>b</sup> |
| Seminal vesicles and coagulating glands (w/liquid) | 1.70 ± 0.04 (11) | 1.18 ± 0.05 (12)*<sup>b</sup> | 0.83 ± 0.05 (10)*<sup>b</sup> | 0.80 ± 0.05 (11)*<sup>b</sup> | 0.47 ± 0.07 (6)*<sup>b</sup> |
| Ventral prostate | 0.71 ± 0.02 (11) | 0.36 ± 0.03 (10)*<sup>b</sup> | No tissue | No tissue | No tissue |
| Dorsolateral prostate | 0.70 ± 0.01 (11) | 0.36 ± 0.02 (10)*<sup>b</sup> | No tissue | No tissue | No tissue |
| Levator ani bulbocavernosus | 1.22 ± 0.02 (11) | 0.59 ± 0.02 (12)*<sup>b</sup> | 0.43 ± 0.02 (10)*<sup>b</sup> | 0.38 ± 0.02 (11)*<sup>b</sup> | 0.25 ± 0.02 (7)*<sup>b</sup> |

*All tissues are nested litter means ± SE with body weight as a covariate. Malformed tissues are not included. There were 11, 12, 10, 11, and 7 litters in the 0, 6.25, 12.5, 25, and 50 mg/kg/day dose groups, respectively.

*Cryptorchid tissues are not included.

*Cryptorchid tissues were not observed in control animals.

*Paired weights.

*Significantly different from control, p < 0.05.

**FIG. 6.** Histological cross section of testes (A, B) and epididymides (C, D) from adult offspring (postnatal day 100) of a dam administered corn oil (A, C) or 25mg flutamide/kg/day (B, D) from gestation day 12 to 21. Normal stages of spermatogenesis are seen in the control testes (A), whereas the flutamide-exposed testes displays abnormal spermatogenesis characterized by decreased to absent spermatogenic cells (all tubules in B). Tubules were lined by Sertoli cells with wispy, pale, eosinophilic cytoplasm. Transition from body to tail in normal prostates in the 6.25 mg/kg/day dose group weighed 50% less than those of controls (Table 2). Microscopically, ducts were decreased in number, dilated and lined by flattened epithelium.
on both an individual and litter basis (Figs. 7A and 7B). Flutamide exposure at the lowest dose resulted in a robust response that reached a maximum or near maximum. The individual dose-response curves for flutamide-induced malformations in the T-dependent tissues were also similar but varied in the maximal response at the 50 mg/kg/day dose level. The curves for malformations of the vas deferens and epididymis were similar in shape and maximal response, as were the dose-response curves for epididymal and testicular weights of noncryptorchid testes. In general, flutamide exposure resulted in a less marked response for these end points as compared with DHT-dependent tissues, and dose levels of 50 mg/kg/day did not induce a maximal effect (Fig. 7C). The litter dose-response curves for seminal vesicle malformations and epididymal and testicular weight displayed comparable relationships. However, flutamide exposure did not induce a maximal response in these end points (Fig. 7D). LABC, seminal vesicle weights, and cryptorchid testes had dose-response curves that were intermediate (in general) between those of the DHT- and T-mediated tissues (Fig. 7E). The dose-response curves for individual pup testicular and epididymal weights were also similar (Fig. 7E). The litter response rate for flutamide-induced cryptorchid testes was similar in shape to dose-response curves observed in DHT-mediated tissues (Fig. 7F and 7A). In contrast, the dose-response curves for flutamide-mediated changes in LABC and seminal vesicle weight were similar to each other and reached similar maximal responses. Likewise, flutamide-mediated affects on epididymal and testicular responses displayed similar response curves (Fig. 7F).

The Ability of AGD on PND 1 to Predict Subsequent Malformation in the Adult, and Association between Retained Nipples and Malformations

Logistic regression was used to determine whether flutamide-mediated decreases in AGD on PND 1 predicted malformation of androgen-dependent tissues in adult animals. Logistic regression analysis of the absence or presence of malformations in a respective tissue versus the AGD of that animal was significant ($p < 0.05$) for all tissues tested. The ROC analysis and subsequent AUC values, which ranged from 0.94 for bulbourethral gland agenesis to 0.85 for seminal vesicle malformations, indicate that changes in AGD are a strong predictor of these subsequent malformations (Table 4). The inverse prediction function was used to calculate the AGD at which 50 and 10% of the animals would have malformations (Table 4). The predicted AGD at which 50 and 10% of the animals would have agenesis of the bulbourethral glands was calculated to be 1.82 and 2.09 mm, respectively. In contrast, the predicted AGD at which 50 and 10% of the male offspring would have seminal vesicle malformations was determined to be 0.94 and 1.21 mm, respectively (i.e., a female AGD) (Table 4).

Adult male animals that had malformations displayed a variable number of nipples (Fig. 8). Animals that exhibited malformations in DHT-dependent tissues (bulbourethral glands, prostate, and external genitalia) displayed 6 or more nipples. In contrast, animals that displayed lesions in T-mediated tissues (seminal vesicles, epididymis, vas deferens) displayed predominately 12 nipples per rat (i.e., a female phenotype) (Fig. 8). The response for cryptorchidism was similar to that of the DHT-dependent tissues.

Interrelationship among Flutamide-Induced Reproductive Tract Malformations

Animals that had displayed a lesion in one DHT-dependent tissue often displayed a lesion in another (i.e., agenesis of the
bulbourethral gland was associated with malformations of the prostate; Table 5). Similarly, animals often displayed lesions in more than one T-dependent tissue (i.e., epididymal agenesis was associated with agenesis of the vas deferens; Table 5). Animals that displayed malformations in T-dependent tissues also exhibited malformations in DHT-dependent development. However, the corollary did not occur (animals that displayed agenesis of the epididymis also displayed malformed bulbourethral glands and/or prostate, but animals that exhibited prostate malformations often did not display epididymal agenesis; Table 5). Cryptorchid testes were associated more often with lesions in DHT-mediated tissues (prostate and bulbourethral glands) than with lesions in the T-dependent tissues.

DISCUSSION

In the present study, male offspring from dams treated with flutamide during the critical period of reproductive development were uniquely identified at birth, and various androgen-mediated end points were examined throughout life. Flutamide-induced alterations in AGD and areola/nipple retention in early postnatal life correlated with a reduction in AGD and retained nipples observed in the adult. In general, flutamide-induced alterations in DHT- and T-dependent development were different and dependent on the androgen sensitivity of that tissue. Tissues that require T (epididymis, vasa deferentia, and seminal vesicles) for development had similar dose-response curves, whereas tissues that require DHT (prostate, external genitalia, and regression of the nipple anlagen) likewise displayed comparable responses. However, the dose-response curves for flutamide-induced changes in cryptorchidism and seminal vesicle weight were intermediate between the dose-response curves for DHT- and T-dependent development, suggesting that proper development of these tissues requires both androgens. Flutamide-induced decreases in AGD predicted subsequent malformations, as evidenced by logistic regression and ROC analysis of malformations versus AGD. However, the AGD in male pups that would predict a 10% incidence of seminal vesicle malformations is equivalent to a female AGD. An almost fully feminized phenotype of 10–12 nipples was observed in animals that had malformations in T-dependent tissues, whereas 6 or more nipples were observed in animals with malformations in DHT-dependent tissues. These data suggest that flutamide-mediated changes in AGD and nipple retention are not sensitive predictors of altered T-mediated development.

Permanence of AGD and Retained Nipples

Flutamide, as well as other antiandrogens, has previously been shown to alter the AGD of male rats and induce retention

TABLE 4

Logistic Regression of Reproductive Tract Malformations on Postnatal Day 100 Versus Anogenital Distance on Postnatal Day 1

<table>
<thead>
<tr>
<th>Malformation</th>
<th>ROC</th>
<th>$M_{50}$</th>
<th>CL$_{Lower}$</th>
<th>CL$_{Upper}$</th>
<th>$M_{10}$</th>
<th>CL$_{Lower}$</th>
<th>CL$_{Upper}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulbourethral glands</td>
<td>0.94</td>
<td>1.82</td>
<td>1.74</td>
<td>1.99</td>
<td>2.09</td>
<td>1.94</td>
<td>2.46</td>
</tr>
<tr>
<td>Prostate</td>
<td>0.96</td>
<td>1.72</td>
<td>1.68</td>
<td>1.79</td>
<td>1.91</td>
<td>1.83</td>
<td>2.07</td>
</tr>
<tr>
<td>Cryptorchidism</td>
<td>0.81</td>
<td>1.71</td>
<td>1.63</td>
<td>1.83</td>
<td>2.28</td>
<td>2.09</td>
<td>2.65</td>
</tr>
<tr>
<td>Epididymis</td>
<td>0.85</td>
<td>1.28</td>
<td>1.20</td>
<td>1.33</td>
<td>1.51</td>
<td>1.47</td>
<td>1.60</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>0.85</td>
<td>1.26</td>
<td>1.16</td>
<td>1.31</td>
<td>1.48</td>
<td>1.44</td>
<td>1.56</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>0.85</td>
<td>0.94</td>
<td>ND</td>
<td>1.56</td>
<td>1.21</td>
<td>ND</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Note. Logistic regression was calculated utilizing JMP 4.0 (SAS Institute, Cary, NC). Presence of a malformation is considered a full response.

*Receiver operator characteristic (ROC) function describes the predictive value of $\times$ (AGD) with a binary outcome (malformation) and is represented as area under the curve (AUC). A value of 1 is highly predictive, whereas a value of 0.5 is not predictive.

*Inverse prediction function of the logistic regression analysis (JMP 4.0, SAS Institute, Cary, NC) that predicts the AGD (mm) at which 50 and 10% of the animals would display the indicated malformation, respectively.

*95% confidence limits of the predicted AGD.

*Not determinable.

FIG. 8. Relationship between flutamide-induced nipple retention and malformations. Animals were exposed to flutamide from gestation days 12 to 21, necropsied on postnatal day 100, and the number of nipples counted.
Table 5: Interrelationship among Malformations Resulting from in Utero Flutamide Exposure

<table>
<thead>
<tr>
<th>Malformation</th>
<th>B-U glands</th>
<th>Prostate</th>
<th>Cryptorchid</th>
<th>Epididymis</th>
<th>Vas deferens</th>
<th>Seminal vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-U glands (175)</td>
<td>94.6</td>
<td>90.4</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Prostate (190)</td>
<td>—</td>
<td>89.7</td>
<td>69.7</td>
<td>21.1</td>
<td>17.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Cryptorchid testes (155)</td>
<td>94.6</td>
<td>90.4</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Epididymis (42)</td>
<td>97.6</td>
<td>76.2</td>
<td>81.0</td>
<td>81.0</td>
<td>9.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Vas deferens (35)</td>
<td>80.0</td>
<td>97.1</td>
<td>11.4</td>
<td>—</td>
<td>11.4</td>
<td>—</td>
</tr>
<tr>
<td>Seminal vesicles (5)</td>
<td>80.0</td>
<td>80.0</td>
<td>80.0</td>
<td>80.0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a Total number of flutamide-exposed animals was 224 (for bulbourethral [B-U] glands it was 196 this lesion was not recorded for the first 28 animals necropsied).
b Total number of malformations observed.
c Percentage of animals responding.

of nipples (Clark et al., 1990; Gray et al., 1999; Hellwig et al., 2000; Imperato-McGinley et al., 1986, 1992; McIntyre et al., 2000; Mylchreest et al., 1998, 1999). However, some researchers have suggested that these changes may at least be partially reversible (Clark et al., 1990; Hellwig et al., 2000). In contrast, studies conducted by Gray et al. (1999) have demonstrated that, on a litter basis, changes seen on PND 1 are also observed in adulthood. In the current study, decreases in AGD and postnatal retention of areolae/nipples in uniquely identified pups correlated strongly with changes in AGD and nipple retention observed at sexual maturity. This issue of permanency of antiandrogen-induced changes in AGD may result from postnatal growth of the perineum and genital area. In the current study, flutamide exposure decreased (relative to control) the AGD 43% on PND 1 but only 29% on PND 100 at the 6.25 mg/kg/day dose level. Therefore, subtle changes in litter mean AGD observed on PND 1 may be masked by within-litter variability on PND 100.

The background incidence of areola/nipple retention on PND 13 in male control pups was low and similar to previously reported values (McIntyre et al., 2000; Mylchreest et al., 1998). However, these areolae/nipples did not develop into identifiable nipples on PND 100. In contrast, 2 control animals did display nipples on PND 100, but neither of these animals was scored as having areolae/nipples on PND 13. To our knowledge, this is the first report demonstrating that control animals display a low incidence of retained nipples. Furthermore, not all flutamide-induced areolae/nipples observed on PND 13 developed into nipples on PND 100. Therefore, some areolae may not fully develop into easily identifiable nipples. Nevertheless, these data demonstrate that the observed changes are permanent for all the dose levels of flutamide employed. Since the definition of a malformation is usually accepted to be a permanent structural change that is either rare or life threatening, it could be argued that the presence of nipples and decreased size of the perineum in adult male rats constitute true malformations and may be used in risk assessment.

Tissue Sensitivity to in Utero Flutamide Exposure

Male offspring exposed to flutamide in utero displayed a spectrum of reproductive tract effects similar to those that have been previously reported (Imperato-McGinley et al., 1992). In this earlier study, in utero flutamide exposure to dose levels as high as 300 mg/kg/day did not affect the weight of descended testes, whereas dose levels of flutamide greater than 75 mg/kg/day decreased epididymal weight and induced malformations of the epididymis and vas deferens (Imperato-McGinley et al., 1992). In contrast to these earlier observations, testicular and epididymal weight was significantly decreased in the 50 mg/kg/day dose group in the current study. In addition, numerous malformations of the epididymis and vas deferens were observed in this dose group. Imperato-McGinley et al. (1992) also demonstrated that in utero exposure to dose levels greater than 18 mg/kg/day induced prostate agenesis and that dose levels higher than 24 mg/kg/day of flutamide induced malformations of the external genitalia. In the current study, prostate agenesis was observed in all dose groups but was more prevalent at the 12.5 mg/kg/day exposure level and higher. Moreover, malformations of the external genitalia were observed in all dose groups. These disparities in experimental findings are likely the result of different routes of flutamide administration to the dam, sc in Imperato-McGinley study versus gavage in the current study (Imperato-McGinley et al., 1992). Flutamide administered orally is rapidly metabolized to the more potent androgen receptor antagonist 4-hydroxy flutamide (Xu and Li, 1998). These findings underscore the importance of route of exposure in determining dose-response relationships for endocrine-active compounds.

Dose-Response Relationships

DHT-dependent processes were similarly affected and more sensitive to flutamide-mediated changes than T-dependent development. These respective responses were similar on both an individual and litter basis. Nipple retention and AGD were associated with other adverse changes in DHT-mediated pro-
cesses such as genital malformations, altered prostate development, and absent bulbourethral glands, suggesting that alterations in these early postnatal end points may serve as markers for altered DHT-dependent reproductive development. In contrast, flutamide-induced malformations of Wolffian duct derivatives occurred predominately at the highest dose level of flutamide administered. These observations confirm and extend the findings of previous investigators demonstrating that flutamide preferentially alters DHT-mediated growth (Imperato-McGinley et al., 1992). Moreover, similar observations have been reported with the pesticides vinclozolin and procymidone, which are also AR antagonists (Gray et al., 1999; Ostby et al., 1999).

The reason for this disparity in the effects of flutamide on T- and DHT-dependent tissues is unclear. The differing sensitivities of these tissues to in utero flutamide exposure may be the result of local androgen and antiandrogen concentrations. During fetal development, local concentrations of T within the differentiating Wolffian duct are higher than in tissues derived from the urogenital sinus (Veyssiere et al., 1982). This differential in androgen concentrations is the result of luminal transport of T from the testis into the differentiating Wolffian duct coupled with increased 17β-hydroxysteroid dehydrogenase metabolism of T within DHT-dependent tissues, relative to the Wolffian ducts (George, 1997). In addition, in utero flutamide exposure does not alter 5α-reductase mRNA expression (Berman et al., 1995), whereas prenatal flutamide exposure has been shown to decrease androgen receptor expression in DHT-dependent tissues (Bentvelsen et al., 1994). Therefore, flutamide-induced malformations may be dependent on the interaction between the local tissue concentrations of T, DHT, flutamide, and androgen receptor levels. Nevertheless, the dramatic decrease in AGD and increased areola/nipple retention seen in the 6.25 mg/kg/day dose group were not associated with a concomitant increase in malformations in T-dependent organ development.

Cryptorchidism and seminal vesicle weight displayed dose-response curves that were intermediate between DHT- and T-mediated tissue responses. The normal descent of the testes has been shown to be dependent on DHT and T (George, 1989; Imperato-McGinley et al., 1992), whereas seminal vesicles are dependent on T for prenatal development and DHT for postnatal growth (Bentvelsen et al., 1995; Shima et al., 1990). Therefore, these intermediate responses likely reflect flutamide-induced effects on the differential androgen requirements of these tissues. Prenatal flutamide decreased LABC weight in a dose-dependent fashion, and these dose-response curves were intermediate between tissues that require T and DHT for development. This observation is interesting since the LABC does not express 5α-reductase, nor is DHT required for pre- and postnatal growth (Blohm et al., 1986; Kumar et al., 1995). Nevertheless, the LABC is the most sensitive indicator of altered T-dependent development measured in this study. However, in utero exposure to the weak AR antagonist linuron has been shown to affect T-mediated development in the absence of effects on LABC weight (McIntyre et al., 2000). Therefore, antiandrogen-induced changes in LABC may indicate altered T-mediated development, but the absence of changes in LABC weight does not preclude malformations in other T-dependent tissues.

In the current study, individual animals often displayed more than one malformation. Epididymal lesions were often associated with agenesis of the vas deferens, whereas bulbourethral gland agenesis was often associated with prostate agenesis and hypospadias. These observations are likely the result of the effects of flutamide on the ontology of these androgen-dependent tissues from the fetal Wolffian ducts, urogenital sinus, and genital tubercle.

**Flutamide-Induced Alterations in AGD and Nipple Retention as Sensitive Predictors/Indicators of Lesions in Adult Animals**

Nipple retention observed in adult male rats exposed in utero to flutamide was associated with malformations in DHT-dependent reproductive tissues. In T-dependent tissues, however, only a fully feminized nipple response was associated with malformations in T-dependent tissues. These data suggest that nipple retention is associated with, and an indicator of, altered DHT-mediated reproductive development. However, counting nipples may not be adequate to predict lesions in T-dependent tissues.

There was a strong association between AGD and subsequent development of reproductive tract malformations, as determined by logistic regression. Moreover, the ROC analysis indicated that AGD is highly predictive of subsequent reproductive malformations. AGD is a sensitive predictor for flutamide-induced reproductive malformations in DHT-mediated tissues, as evidenced by the predicted AGD that would be required for 10% of the animals to display a respective lesion. However, a fully or near completely feminized AGD would be required to predict a 10% incidence of a malformation in T-dependent reproductive tissues. Although flutamide-induced changes in AGD predict subsequent malformation of the reproductive tract, T-dependent tissues require almost a female-like AGD before malformations are detectable. Therefore, AGD would not be the most sensitive end point for indicating antiandrogen-induced malformations in T-dependent tissues. Moreover, studies examining the effects of in utero exposure to linuron have demonstrated that lesions in T-dependent tissues may occur in the absence of significant changes in AGD or nipple retention (McIntyre et al., 2000).

In summary, the current study demonstrated that exposure from GD 12 to 21 to the androgen receptor antagonist flutamide resulted in permanent alterations in AGD and retention of nipples in male offspring. Changes in AGD and areola/nipple retention in the early postnatal period correlated with altered androgen-dependent development observed in the...
adult. DHT-mediated development of the prostate and external genitalia was more sensitive to flutamide-exposure than T-dependent development of the epididymides, *vasa deferentia*, and seminal vesicles. However, the AGD on PND 1 necessary to predict malformations in T-dependent tissues would be female-like. Moreover, a female nipple phenotype was required before T-dependent reproductive development was adversely affected. These findings indicate that antiandrogen-induced changes in AGD and areola/nipple retention whilst being sensitive to DHT-mediated development are insensitive indicators of altered T-dependent reproductive development.

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