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Timing of Prenatal Androgen Exposure: Anatomical and Endocrine Effects on Juvenile Male and Female Rhesus Monkeys

Rebecca A. Herman,*[,1] Benjamin Jones,* David R. Mann,† and Kim Wallen*

**Yerkes Regional Primate Research Center, Department of Psychology, Emory University, Atlanta, Georgia 30322; and †Department of Physiology, Morehouse School of Medicine, Atlanta, Georgia 30310*

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Prenatal androgen shapes genital differentiation. In humans, genital anatomy determines sex of rearing and subsequent behavioral development. Rhesus monkey genital anatomy and neuroendocrine function are sexually differentiated, and behavioral development occurs in a complex social environment. We investigated prenatal hormonal influences on sexual differentiation by suppressing or increasing androgens in male and female rhesus monkeys. Pregnant multiparous female rhesus monkeys received 35–40 days of testosterone enanthate (TE) treatment, androgen antagonist (flutamide, FL) treatment, or vehicle starting on gestation day (GD) 35 or 40 (early) or GD 110 or 115 (late). Exogenous androgen increased neonatal LH secretion in females when given early and altered female genital differentiation when administered either early or late. TE treatment, early or late in gestation, had no measurable effects on male genital differentiation or neuroendocrine function. Early FL treatment, however, radically altered male genital differentiation, producing in two cases males with a urethral opening separate from the glans. In females, early FL treatment produced detectable alterations in genitalia consistent with a reduced exposure to prenatal androgen, suggesting that female rhesus monkeys are naturally exposed prenatally to meaningful levels of T. Late FL treatment reduced male penis size and increased neonatal T secretion, but had no effect in females. This is the first study to block endogenous prenatal testosterone in rhesus monkeys, thereby altering sexual differentiation. These findings illustrate the complexity of prenatal influences on anatomical and neuroendocrine development. The relationship between the anatomical changes reported here and sex differences in behavior is currently under investigation. **© 2000 Academic Press**

Key Words: **prenatal androgen; rhesus monkeys; genital differentiation; growth; neonatal endocrinology; sexual differentiation; flutamide.**

Genital anatomy is the principal determinant of sex of assignment in newborn humans (Diamond and Sigmundson, 1997). The dependence of genital masculinization on circulating prenatal androgens makes it a sensitive external marker of prenatal exposure to androgen. Human males that are not exposed to sufficient levels or appropriate forms of androgens may be born with hypospadia, micropenis, ambiguous, or completely feminine genitalia. This condition results, most commonly, from androgen insensitivity syndrome (AIS; Sinnecker, Hiort, Nitsche, Holterhus, and Kruse, 1997) or 5α-reductase deficiency (Wilson, Griffin, and Russell, 1993; Fratianni and Imperato-McGinley, 1994). Human females exposed to excess prenatal androgens due to congenital adrenal hyperplasia (CAH; White, New, and Dupont, 1987) or exogenous androgenic compounds (Wilkins, 1960) are born with varying degrees of genital virilization, including clitoral hypertrophy, labial fusion, displacement of the urethral opening, or development of a penis. Prenatal or neonatal androgen also influences postnatal growth in mammals (Tarttelin, Shryne, and Gorski, 1975; DeHaan, Berger, Kesler, McKeith, Faulkner, and Cmarik, 1988; Gill and Hosking, 1995) and alters neuroendocrine function (Clarke and Scaramuzzi, 1978; Mann, Gould, Collins, and Wallen, 1989; vom Saal, 1989; Abbott, Dumesic, Eisner, Kemnitz, and Goy, 1997).

[1] To whom correspondence should be addressed. E-mail: rherman@emory.edu.

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The relationship between these physical indicators of early androgen exposure and sexual differentiation of behavior remains unresolved (Goy, Bercovitch, and McBrair, 1988; Wallen, 1996) and is the ultimate purpose of the present study. This paper focuses on the anatomical and neuroendocrine effects of manipulating prenatal androgens in rhesus monkeys and their potential impact on behavior.

In primates, the genitalia of both males and females develop from common undifferentiated anatomical structures that are shaped through a variety of morphogenic stages. Under the influence of androgens, the genitalia differentiate along males lines, while in the absence of androgens, the genitalia differentiate in a female pattern. In both sexes, a urethral groove forms along the interior surface of the genital tubercle, and labioscrotal swellings develop. Masculinization occurs as the genital tubercle develops into a phallus and the labioscrotal swellings become a scrotum. In females, the genital tubercle becomes the clitoris and the labioscrotal swellings form the labia majora. Jirásek (1976) describes the development of external genitalia in humans in detail. In rhesus monkeys, differentiation of the external genitalia occurs beginning at about gestational day (GD) 55–60 (Prahalada, Tarantal, Harris, Ellsworth, Clarke, Skiles, MacKenzie, Kruk, Ablin, Cukierski, Peter, vanZwieten, and Hendrickx, 1997), and is complete by approximately GD 80 (Abbott *et al.*, 1997). Thus, most of the critical steps in genital differentiation in primates occur prenatally.

Variation in prenatal androgen exposure has definitive effects on genital morphology in mammalian species. Natural variation in androgen levels due to intrauterine position in rats and mice (vom Saal, 1989) and treatment with exogenous testosterone in rats and guinea pigs (Phoenix, Goy, Gerall, and Young, 1959; Rhees, Kirk, Sephton, and Lephart, 1997) have been shown to masculinize genitalia of females. Blocking endogenous prenatal androgen in males likewise affects genital differentiation. The 5α-reductase inhibitor finasteride partially feminized the external genitalia of male rats while the antiandrogen flutamide completely feminized male external genitalia (Imperato-McGinley, Sanchez, Spencer, Yee, and Vaughan, 1992). In females, similar treatment with antiandrogens increased sensitivity to estradiol for inducing lordosis behavior, indicating apparent defeminizing effects of endogenous androgens in rat females (Gladue and Clemens, 1978). When pregnant guinea pigs were treated with the antiandrogen flutamide, the female offspring also displayed increased sensitivity to estradiol for the induction of lordosis behavior, although there were no measurable genital effects of this treatment (Thornton, Irving, and Goy, 1991). Male guinea pigs whose mothers were exposed to antiandrogens during pregnancy exhibited pronounced alterations of their external genitalia with leaf-shaped, nontubular phalluses, and, sometimes, developed vaginal membranes (Goldfoot, Resko, and Goy, 1971; Thornton *et al.*, 1991).

Our knowledge of primate genital differentiation stems from studies in which female fetuses were exposed to exogenous androgens in the maternal circulation. The effects of prenatal androgen treatment on genital differentiation in females depend upon the timing and duration of the treatment. In rhesus monkeys, exposure to 10 mg of testosterone propionate (TP) daily from 40 to 64 days of gestation virilized female offspring, resulting in complete scrotal and penile development. In contrast, later prenatal exposure (GD 115–139) produced only transient clitoral hypertrophy that disappeared soon after birth (Goy *et al.*, 1988). Dihydrotestosterone propionate (DHTP) treatment was less effective than TP in masculinizing female genitalia. Females exposed daily to 10 mg of DHTP prenatally had only partially masculinized genitalia, typically with a conspicuous median raphé and the scrotum rostral to the small penile shaft (Goy, 1981). Such results suggest that the timing of exposure to androgen has marked effects on the degree of genital masculinization. Much less evidence has been developed by manipulating endogenous androgens in males. Oral administration of 2 mg/kg BW/day of finasteride, a 5α-reductase inhibitor, to pregnant rhesus monkey females from GD 20 to GD 100 produced male offspring with a small phallus, a prominent midline raphé, an underdeveloped scrotum, and preputial adhesions of the foreskin to the glans penis (Prahalada *et al.*, 1997). Similarly, in male rats blocking the conversion of testosterone (T) to DHT produced limited effects on male genital differentiation, unlike the more robust effects produced by androgen-receptor antagonists (Imperato-McGinley *et al.*, 1992). Neonatal administration of a gonadotropin-releasing hormone (GnRH) agonist, which after an initial increase suppresses gonadal hormone release, to male rhesus infants from 1 week to 6 months of age resulted in smaller penis length compared to controls and delayed detachment of the glans from the prepuce of the penis (Brown, Nevison, Fraser, and Dixson, 1999). However, these effects were not evident by 1 year of age.

The time course of neural differentiation is quite

different from that of differentiation of the genital anatomy. Proliferation of cortical neurons is not completed in some areas of the macaque brain until GD 100 (Rakic, 1988), and synaptogenesis of these neurons occurs even later in gestation and continues through the first 2 postnatal months (Bourgeois, Goldman-Rakic, and Rakic, 1994; Granger, Tekaia, Le Sourd, Rakic, and Bourgeois, 1995). Thus, it is possible that alterations in the prenatal hormone environment occurring late in gestation may affect neural differentiation without altering the appearance of the genitalia. Similarly, alterations in genital differentiation may be unaccompanied by alterations in neural differentiation. In rhesus monkeys there is one report of behavioral masculinization without detectable genital masculinization (Goy *et al.*, 1988). Whether this reflects a principle of primate sexual differentiation remains to be demonstrated.

Neonatal sex differences in endocrine function of male and female rhesus monkeys are well documented. Gonadally intact infant males show elevated levels of both follicle-stimulating hormone and luteinizing hormone (LH) during the first 3 or 4 months of life, and the testes produce high levels of testosterone during the same period (Frawley and Neill, 1979; Mann, Davis-DaSilva, Wallen, Coan, Evans, and Collins, 1984). These hormones fall to undetectable levels by 6 months and remain there until puberty. During the same neonatal period, males will respond to exogenous GnRH with increased gonadotropin release (Monroe, Yamamoto, and Jaffe, 1983; Mann *et al.*, 1984), but by 6 months this response too is eliminated. In males, neonatal gonadectomy results in a circhoral pattern of LH release that mimics that observed in adult castrated males (Plant, 1986). Infant females also have elevated gonadotropin levels and respond to exogenous GnRH with increased gonadotropin secretion during the first months of life (Monroe *et al.*, 1983). However, serum LH levels of infant females are below those of adult females even though the infants lack the high circulating levels of 17β-estradiol that adult females experience (Plant, 1986). Thus, the negative feedback regulation of gonadotropin secretion in neonate females does not appear to be dependent on gonadal steroids as it is in adult females and infant and adult males. Plant (1986) suggests that the infantile females may be incapable of producing the circhoral pattern of LH release typical of ovariectomized adult females because of incomplete development of the hypothalamic GnRH pulse generator and that this sex difference among infants is likely caused by prenatal effects of testicular hormones on the developing brain. We measured serum levels of LH and testosterone and response to a GnRH injection to investigate the effect of an altered prenatal androgen environment on neonatal endocrine function.

Altering the neonatal hormonal environment can have permanent effects on reproductive function. Blocking the neonatal surge of testosterone in male rhesus monkeys with a GnRH agonist can delay puberty (Mann *et al.*, 1989), produce some alterations in central nervous system centers involved in GnRH secretion (Mann, Akinbami, Gould, Tanner, and Wallen, 1993), and may reduce adult male sexual motivation (Eisler, Tannenbaum, Mann, and Wallen, 1993). Its effect in juvenile behavior is less clear with one instance of alteration in mother–infant interaction (Wallen, Maestripieri, and Mann, 1995) that was not found in a study from another laboratory using a different GnRH analogue treatment (Nevison, Brown, and Dixson, 1997).

The objective of the current study was to investigate the effects of treatment with testosterone or an androgen antagonist during early or late gestation on external genital differentiation, somatic growth, and endocrine activity during the first 18 months of life in male and female monkeys. Such data are important for understanding the normal process of sexual differentiation and the relationship between physiological effects of androgens and their effects on sexual differentiation of behavior during development and adulthood.

METHODS

Animals

Subjects were the 65 offspring of time-mated female rhesus monkeys (*Macaca mulatta*) from two (85- to 100-animal) multimale, multifemale social groups at the Field Station of the Yerkes Regional Primate Research Center. Subjects were housed with their natal group in 25 m $\times$ 25 m outdoor compounds with attached temperature-controlled indoor quarters. Water was continuously available, and the monkeys were fed monkey chow twice daily (Harlan Teklad, Madison, WI), supplemented once per day with fruits and vegetables. Subjects were created in two cohorts over two consecutive birth seasons (March–June of each year). All research was conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals.*

The method used to produce timed mating has been

TABLE 1
Treatment Nomenclature and Numbers of Subjects Surviving to at least 2 Months for Two Cohorts of Rhesus Monkeys

Treatment & timing	Female	Male	Female				Male			
			Androgen		Flutamide		Androgen		Flutamide	
	Vehicle control combined	Vehicle control combined	Early	Late	Early	Late	Early	Late	Early	Late
Abbreviation	VCF	VCM	EAF	LAF	EFF	LFF	EAM	LAM	EFM	LFM
Cohort 1	5	0	4	5	4	1	4	1	2	3
Cohort 2	2	7	2	2	3	6	2	4	5	3
Total	7	7	6	7	7	7	6	5	7	6

described in detail elsewhere (Zehr, Tannenbaum, Jones, and Wallen, submitted for publication). Briefly, it involved observations of female sexual initiation 3 to 5 days per week and verification of pregnancy using ultrasonic visualization. The last day in which a female showed consistent interaction with adult males was considered the day of conception. This behavioral estimate of conception date was compared to an estimate based on ultrasonic visualization. For the ultrasonic visualization estimate, day of conception was derived from visualization after day 20 of pregnancy using a previously published regression of maximum fetal length against gestation day (Tarantal and Hendrickx, 1988). Over a total of the 65 pregnancies producing the subjects of this study, the two estimates were within 5 days of each other in 75% of the cases and within 10 days of each other in 89% of the pregnancies. Pregnancies in which the two estimates were disparate by over 5 days were evenly distributed across treatment groups.

Treatment Injection Protocol

Multiparous females were assigned randomly to one of six treatment groups, taking the social rank of each female's matriline into consideration such that there was a comparable representation of treatments across matrilines. Pregnant females received supplementary androgen (20 mg/week of testosterone enanthate (TE) im at a concentration of 100 mg/ml dissolved in sesame oil), an androgen-receptor blocker (30 mg/kg twice daily of flutamide im dissolved in dimethyl sulfoxide, at a concentration of 500 mg/ml), or twice daily DMSO vehicle injections (all reagents from Sigma Chemical Co., St. Louis, MO). Treatment was started either early (estimated GD 40 in cohort 1 or GD 35 in cohort 2) or late (GD 115 in cohort 1 or GD 110 in cohort 2) in gestation (~170 days) and continued for 35 (cohort 1) or 40 (cohort 2) days.

For treatment, individual females were trained to leave the outdoor compound and enter the indoor housing area on command. Once inside, females were trained to enter a small cage for transfer to a modified housing cage with a moveable back wall. Females were briefly restrained and injected with TE, flutamide, or vehicles. Injections were administered twice daily between 7:30 and 11:30 AM and between 1:30 and 6:00 PM. Androgen-treated pregnant females received TE injections on the morning of days 1, 7, 14, and 21 of the 35- to 40-day treatment period. These females received 0.25 cc of DMSO twice daily on all other days of treatment and in the afternoon of days when they received morning injections of TE. Vehicle control pregnancies early and late in gestation were combined for this study. This produced eight experimental and two vehicle control groups of infants as shown in Table 1.

Maternal Hormonal Assays

Selected blood samples were collected from the androgen- and flutamide-treated mothers throughout their treatment periods. Five milliliters of saphenous vein blood was taken from unanesthetized females prior to the morning injections. In cohort 1, blood samples were collected from androgen-treated females before their morning injections on treatment days 3, 7, 21, 30, 35, 41 or 42, and 49 both early and late in gestation. No samples were taken in cohort 1 from flutamide-treated females. In cohort 2, blood samples were taken from androgen- and flutamide-treated pregnancies on treatment days 1, 3, 7, 21, 28, and 35 early and late in gestation. Blood was also drawn from androgen-treated females on treatment days 41 or 42 and 49. Blood samples were taken to monitor the testosterone levels in treated individuals, and no samples were collected during the treatment period from control pregnancies.

Blood samples were centrifuged, and serum was frozen at −20°C. until assayed. Maternal T levels for androgen-treated pregnancies were assayed with radioimmunoassay using kits produced by Diagnostic Products Corp. (Los Angeles, CA) in the laboratory of Dr. David Mann at the Morehouse School of Medicine. The testosterone assay had a sensitivity of 0.20 ng/ml with an intra-assay coefficient of variation (CV) of 5% and an inter-assay CV of 6%. Maternal T from flutamide-treated pregnancies was assayed by the Yerkes Regional Primate Research Center Assay Services with the same radioimmunoassay kit, with a sensitivity of 0.05 ng/ml, intra-assay CV of 4.3%, and inter-assay CV of 8.2%.

Physical Measurements

Subject monkeys were briefly removed from their group within the first 4 days of life, at 2 weeks of age, and then monthly until 6 months of age for collection of blood samples and photographs of genitalia. The mothers of the subjects were trained to enter an indoor housing area while carrying their infants. Once inside, mother and infant entered a small transfer box and moved to a modified housing cage with a moveable back wall. Initially, the moveable wall was used to restrain the mother against the cage front and the infant was grasped though a 9.5 cm × 9.5 cm opening and removed by the researchers. Once accustomed to this procedure, the mothers would typically push the infants off their bodies, allowing the infants to be easily removed by the investigators. By approximately 1 year of age, the young monkeys went through this handling procedure independent of their mothers. At 2, 8 or 9, 12, and 18 months of age, the animals were immobilized with ketamine (0.1 mg/kg, supplemented as necessary) for morphometric measurements. Each morphometric measurement was taken twice. If the two measurements differed by more than 5%, the measurement was repeated. The average of the two measurements was used for data analysis. The following morphometric measurements were recorded: weight, crown–rump length (the distance (in centimeters) from the crown of the subject's head to its ischial callosities using Ross Knee Height calipers (Ross Products, Columbus, OH); crown–heel length (the distance (cm) from the crown of the head to the heel of the extended left leg); tibia length (the length of the left tibia from the notch where the patella and tibia meet at the knee to the notch at the ankle where the tibia ends, using vernier calipers (Fisher Scientific, Pittsburgh, PA)); chest circumference (the distance around the subject's chest across the nipples, measured with a fabric string drawn snugly under the monkey's arms); abdomen circumference (the distance around the subject's abdomen across the navel, using the same procedure as for the chest measurements).

Calculated morphometric measurements. The ratio of crown–rump length to crown–heel length (CR/CH ratio) was determined for all subjects. This measurement illustrates treatment effects on body proportions (Merlob, Sivan, and Reisner, 1986). The ratio of abdomen circumference to chest circumference (Abdomen/Chest ratio) was also calculated. Abbott *et al.* (1997) found that prenatal androgen exposure in female rhesus monkeys alters fat distribution as adults such that greater adiposity of the abdominal area is prevalent. The abdomen/chest ratio should illustrate any effects on abdomen size relative to body size.

Female genital measurements. The following measurements were recorded: anus to vagina (AV) length (the distance from the center of the anal opening (A) to the posterior vaginal fourchette (V) measured with vernier calipers); vagina to clitoris (VC) length (the distance from the fourchette to the base of the clitoris (C)); anus to clitoris (AC) length (the distance from the center of the anal opening to the base of the clitoris). The ratio of AV length to AC length (AV/AC ratio) was also determined for females. This measurement has been suggested to vary with prenatal androgen exposure in humans and does not covary with age, body weight, or size (Callegari, Everett, Ross, and Brasel, 1987).

Male genital measurement. Penis length was measured as the distance from the base of the unstretched penis on the upper side to the tip of the exposed glans, using vernier calipers. The foreskin was pulled back to expose the glans and then released before the measurement was taken.

Genital Masculinization

The masculinization of male subjects' genitalia was scored on a scale of 0 to 18 (Table 2). Scoring was based upon photographs taken during the first 18 months of life. Zero represented "typical" female genitalia and "typical" males were expected to score 17. Phallus size was classified as small, developed, or enlarged based upon the average of the measurements taken per individual (25 males had four measurements, 3 had three measurements, and 1 had two measurements; 2 males with only one measurement were not scored). Phalluses averaging over half of a

TABLE 2
Criteria for Assigning Genital Masculinization Scores in Male Rhesus Monkey Infants

Criteria	Score	Typical male score
Median raphé presence		
Ischial callosity midline raphé, no or very small scrotum	1	
Prominent scrotal and ischial callosity raphé	2	
Prominent scrotal raphé only	3	
No prominent raphé, scrotal suture line	4	4
Scrotal development		
Small cephalic scrotum	2	
Scrotum with integral phallus, scrotum surrounds phallus	3	
Small scrotum caudal to phallus	4	
Caudal pendulous scrotum	5	5
Phallic development		
Enlarged clitoris with separate urinary opening	1	
Glans or small tubular phallus with separate urinary opening	2	
No real phallus, but enlarged glans with urethral opening	3	
Small tubular phallus with urethral groove	4	
Small tubular phallus with integral urinary opening	5	
Developed phallus with integral urinary opening	6	6
Enlarged phallus	7	
Female features		
No vaginal opening	1	1
No labial folds	1	1
Score		17

Note. Higher scores indicate greater masculinization.

standard deviation less than the average of control males were classified as small. Developed phalluses fell within half of a standard deviation from the average phallus size of control males. An average over half of a standard deviation greater than the average for the control males was classified as enlarged.

Subject Hormonal Assays

Blood samples were taken from all subjects at 2 weeks, monthly from 1 to 6 months, at 8 or 9 months, at 12 months, and at 18 months. At 2, 8 or 9, and 18 months, subjects were challenged with an iv bolus of 50 ng/kg BW of GnRH. Blood samples were drawn before and at 60 min after the GnRH injection.

Testosterone was assayed as described above for androgen-treated maternal blood samples. LH was assayed using the mouse interstitial cell-testosterone bioassay as modified by Steiner and Bremner (1981). The LH assay had a sensitivity of 4.0 ng/ml with an intra-assay coefficient of variation (CV) of 8% and an inter-assay CV of 17%. The data are expressed in terms of the monkey pituitary WP-XV-20 LH standard. The pre- and postchallenge samples from a given monkey were run in the same assay each time, and different treatment groups were present in each assay as the assays were done chronologically by time of sample collection.

Statistical Analysis

All statistical analyses were conducted with SPSS for Windows (Version 8.0, SPSS Inc.). A P value of less than 0.05 was considered statistically significant, and values of P between 0.05 and 0.10 are also reported to indicate trends.

To analyze the maternal T levels during pregnancy, a t test compared T levels in androgen-treated and flutamide-treated mothers, and 2×2 ANOVAs with day of treatment and timing of treatment as factors were conducted separately for flutamide- and androgen-treated pregnancies. Morphometric data and female genital measurements were analyzed using multivariate analysis of covariance (MANCOVA) with age as a covariate. Pairwise comparisons between treatment groups were based upon estimated marginal means, thereby eliminating age effects. For morphometric measurements of all 10 treatment groups, pairwise comparisons were conducted between control groups and experimental treatment groups only. Penis length was analyzed with a 2×2 ANOVA with age and treatment group as factors, and *post hoc* Tukey

tests compared individual treatments. A one-way ANOVA and *post hoc* Tukey tests were used in the analysis of genital masculinization scores. To analyze the relationship between variation in genital masculinization and possible causes of the variation, bivariate correlations were calculated. Serum levels of LH and T were analyzed separately in males and females with a 2 × 2 ANOVA with age and treatment groups as factors. Comparisons between ages or treatment groups were conducted with *post hoc* Tukey tests. In the analysis of the response to a GnRH challenge, the percentage change in LH and T was calculated [((postchallenge value − prechallenge value)/prechallenge value) × 100%]. Differences in response by treatment group were analyzed with one-way ANOVAs.

RESULTS

Maternal Androgen Levels during Treatment

Testosterone levels in androgen-treated mothers during treatment ranged from 2.4 to 21.7 ng/ml (sampled immediately preceding the week's injection of TE) and were all substantially above physiological T levels seen in normal pregnancies. The T levels of flutamide-treated mothers were much lower, ranging from less than 50 to 480 pg/ml. A comparison of the lowest T value for androgen-treated mothers collected during the treatment period and highest T value for flutamide-treated mothers during treatment showed that the groups differed significantly ($t_{23.29} = 15.49$, $P < 0.001$). Testosterone levels did not differ with fetal sex for either prenatal treatment (data not shown). For females receiving androgen injections, maternal T levels differed by timing of treatment (early versus late gestation; $F_{1,140} = 7.04$, $P = 0.009$), by day within treatment ($F_{7,140} = 48.97$, $P < 0.001$), and by an interaction between the two factors ($F_{7,140} = 3.27$, $P = 0.003$) such that T levels during the first week of treatment were lower in mothers treated late in pregnancy than in mothers treated early in pregnancy (data not shown). Timing of treatment within gestation did not affect T levels in flutamide-treated pregnancies. However, testosterone levels tended to be higher from the start of the fourth week through the end of the treatment than they were through the start of the second week of treatment in flutamide-treated mothers (planned contrast, $t_{90} = -3.643$, $P = 0.001$).

Somatic Growth

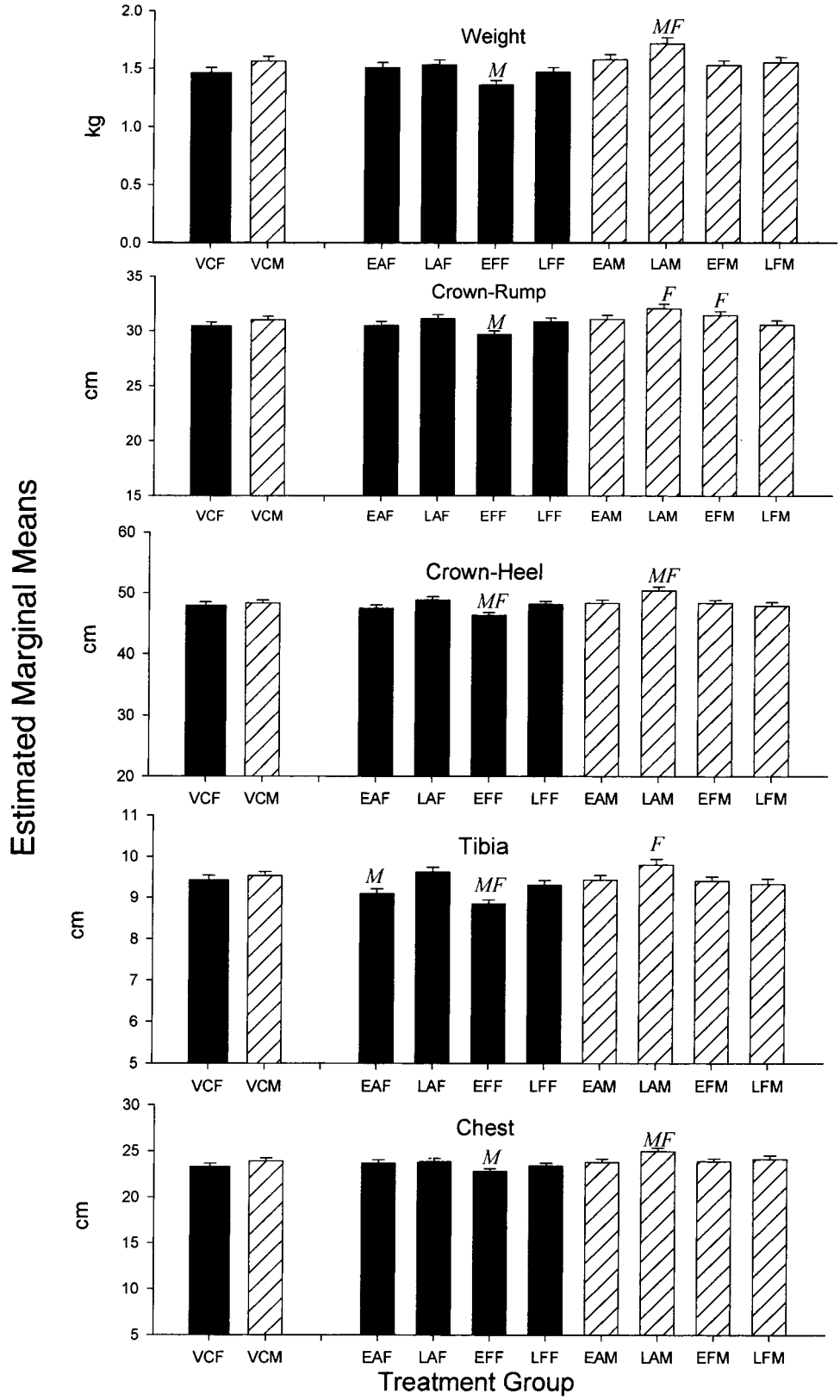
Age of subject significantly affected each of the morphometric measurements in a multivariate analysis of weight, crown–rump length, crown–heel length, tibia length, chest circumference, and abdomen circumference. However, no age by treatment interaction existed for any measurement ($P > 0.10$). Therefore, a MANCOVA with age as a covariate was used to analyze the morphometric data. The multivariate test indicated that the treatment means were not equal (Roy's Largest Root, $P < 0.001$). As presented in Fig. 1, treatment significantly affected weight ($F_{9,222} = 4.03$, $P < 0.001$), crown–rump length ($F_{9,222} = 2.96$, $P = 0.002$), crown–heel length ($F_{9,222} = 3.29$, $P = 0.001$), tibia length ($F_{9,222} = 5.42$, $P < 0.001$), and chest circumference ($F_{9,222} = 2.47$, $P = 0.011$). Treatment did not significantly affect abdomen circumference.

Pairwise comparisons using estimated marginal means showed that control males and control females did not differ significantly in weight. LAMs weighed more than control males and females (VCMs, $P = 0.021$; VCFs, $P < 0.001$), and EFFs weighed less than control males ($P < 0.001$).

Control males and control females did not differ in crown–rump length ($P < 0.10$). However, EFFs had shorter crown–rump lengths than control males ($P = 0.005$), and both LAMs and EFMs had longer crown–rump lengths than control females ($P = 0.006$ and $P = 0.048$, respectively). Controls in the two sexes also did not differ in crown–heel length ($P > 0.10$). Crown–heel length of LAMs was greater than both control females ($P = 0.005$) and control males ($P = 0.013$). EFFs were smaller than both control females ($P = 0.032$) and control males ($P = 0.004$).

Tibia length did not differ between control males and control females ($P > 0.10$). EFFs had smaller tibias than both control females and control males ($P < 0.001$ for both), and EAFs had smaller tibias than control

FIG. 1. Estimated marginal means ± SE for somatic measurements in rhesus monkeys that differed significantly by treatment group. *M*, significantly different from VCM in pairwise comparisons ($P < 0.05$). *F*, significantly different from VCF in pairwise comparisons ($P < 0.05$). VCF, vehicle control female; VCM, vehicle control male; EAF, early gestation androgen-treated female; LAF, late gestation androgen-treated female; EFF, early gestation flutamide-treated female; LFF, late gestation flutamide-treated female; EAM, early gestation androgen-treated male; LAM, late gestation androgen-treated male; EFM, early gestation flutamide-treated male; LFM, late gestation flutamide-treated male.


Estimated Marginal Means
Weight
kg
2.0
1.5
1.0
0.5
0.0
MF
M
Crown-Rump
cm
35
30
25
20
15
F
F
M
Crown-Heel
cm
60
50
40
30
20
MF
MF
Tibia
cm
11
10
9
8
7
6
5
M
MF
F
Chest
cm
30
25
20
15
10
5
MF
M
VCF VCM EAF LAF EFF LFF EAM LAM EFM LFM
Treatment Group

males ($P = 0.007$). LAMs had longer tibias than control females ($P = 0.039$).

Control males and control females did not differ in chest circumference ($P > 0.10$). Again, LAMs were the largest, with chest circumferences greater than both control females ($P = 0.003$) and control males ($P = 0.046$). EFFs had smaller chest circumferences than control males ($P = 0.011$).

A separate multivariate analysis was conducted for the derived measurements, CR/CH ratio and abdomen/chest ratio. As age of subject significantly affected the measurements, a MANCOVA with age as a covariate was used. The multivariate test demonstrated that treatment groups differed (Roy's Largest Root, $P = 0.006$). The abdomen/chest ratio did not differ by treatment ($P > 0.10$), but prenatal treatment significantly affected the CR/CH ratio ($F_{9,222} = 1.96$, $P = 0.045$, data not shown). Pairwise comparisons between the experimental treatment groups and the control groups indicated that EFMs had a larger CR/CH ratio than control subjects of either sex ($P = 0.004$ for both VCMs and VCFs; data not shown).

Genital Anatomy of Females

Qualitative observations and photographs of the female genitalia revealed no obvious treatment effects. Two EAFs had possible labial fusion on the day of birth, but by 1 month, no fusion was noted. Clitoral hypertrophy was observed at 2 weeks in two females, one EAF and one EFF, and hypertrophy was not visible by 2 months.

In a MANOVA with age and treatment group as fixed factors, age affected all female genital measurements except the AV/AC ratio, and no age by treatment interactions were present. Thus, the data were again analyzed with a MANCOVA with age as a covariate. The multivariate test indicated that the treatment means were not equal (Roy's Largest Root, $P < 0.001$). Neither AV length nor AC length differed according to treatment ($P > 0.10$). Prenatal treatment significantly affected VC length ($F_{4,119} = 3.98$, $P = 0.005$; data not shown) and the AV/AC ratio ($F_{4,119} = 3.50$, $P = 0.010$; Fig. 2a).

Pairwise comparisons using estimated marginal means indicated that VC length was greater in EAFs than in both VCFs ($P = 0.002$) and LFFs ($P = 0.005$), but did not differ from the other female treatment groups. EFFs also had a longer VC length than both VCFs ($P = 0.008$) and LFFs ($P = 0.022$), but did not differ from other female treatment groups. The AV/AC ratio was largest in VCFs. Control females had a significantly larger ratio than EAFs ($P = 0.020$), LAFs ($P = 0.020$), and EFFs ($P = 0.003$). EFFs had the smallest ratio, which was significantly smaller than the LFFs also ($P = 0.012$).

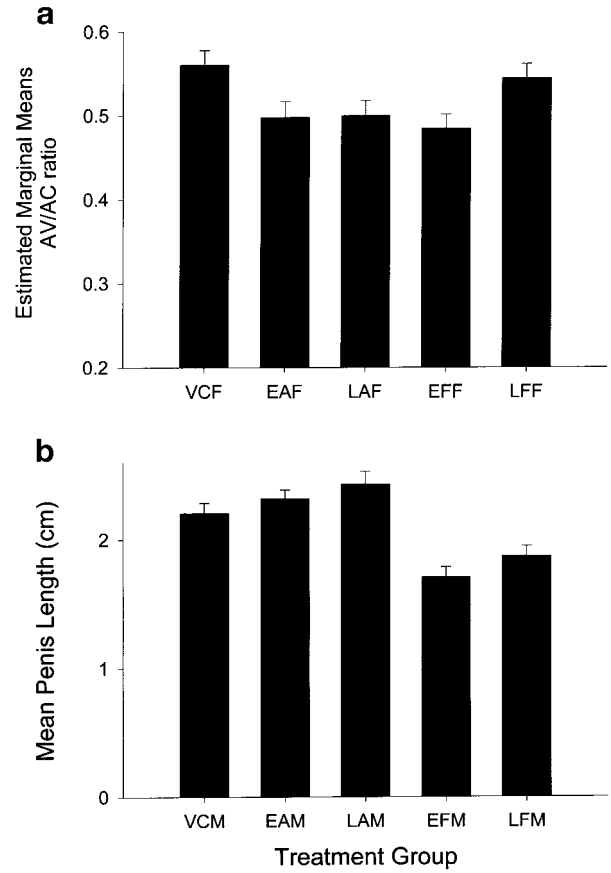


FIG. 2. (a). Estimated marginal means ± SE for AV/AC ratio in female rhesus monkey subjects by treatment group. VCF > EAF, LAF, EFF; LFF > EFF ($P < 0.05$ for all). (b). Mean penis length ± SE of male rhesus monkey subjects by treatment group. LAM, EAM, VCM > LFM, EFM ($P < 0.05$). Abbreviations as in Fig. 2.

Genital Anatomy of Males

A general factorial ANOVA indicated that the length of the unstretched penis was unaffected by age, and age and treatment did not interact ($P > 0.10$). However, treatment affected penis length (Fig. 2b, $F_{4,93} = 13.61$, $P < 0.001$). LFMs and EFMs had significantly smaller penises than VCMs, EAMs, and LAMs (*post hoc* Tukey test; LFM: VCM, $P = 0.028$; EAM, $P = 0.002$; LAM, $P < 0.001$; EFM: $P < 0.001$ for each). For those mothers in which estimates of T were available, correlations between maternal T on 5 days during

treatment and penis length within both the flutamide-treated and the androgen-treated groups were calculated. On day 35 of treatment, $r = 0.458$ ($n = 10$) for androgen-treated pregnancies and $r = -0.573$ ($n = 8$) for flutamide-treated pregnancies, but no correlations were significant ($P > 0.10$ for all).

While the penises of the LFMs were smaller than those of controls, the overall masculinization of the genitalia was normal. In the EFMs, however, abnormal development of the genitalia was apparent. A one-way ANOVA of the scores for male genital masculinization showed significant treatment effects ($F_{4,24} = 18.78$, $P < 0.001$). EFMs had significantly lower genital masculinization scores than the other four groups (*post hoc* Tukey test, $P < 0.001$ for all). However, genital masculinization scores for EFMs showed great variation and ranged from 6 to 16. In comparison, all LFMs had genital masculinization scores of 16, control males had scores of 16 to 18, and all EAMs and LAMs had scores of 17 or 18 as a result of their larger penis length. What is perhaps most surprising is the large variation seen in the EFMs, from apparently normal genital appearance to a hypospadic penis caudal to the scrotum with a prominent median raphé. The most extensively modified EFMs had no apparent penile shaft and a urethral opening completely separated from the glans. One of these died at less than 2 weeks of age, preventing his inclusion in any of the analyses. Autopsy examination of the reproductive structures of this male demonstrated that the urethra was clearly separated from the clitoris-like phallic structure in a female-typical orientation. Normal-appearing testes, prostate, and seminal vesicles were found internally. Thus, early prenatal flutamide treatment did not apparently inhibit Wolffian duct development. No evidence of any Mullerian duct derivatives was found, and there was no evidence of a vaginal vault around the urethra. Figure 3 presents the genitalia of three EFMs that illustrate the variation in genital development. The source of this extreme variation is not known, but several possibilities can be ruled out.

Our method of determining conception date (Zehr *et al.*, submitted for publication) may have produced differences between the presumed GD when treatments were started and the actual GD. However, we could not detect a significant correlation between the seven EFMs' genital masculinization scores and the GD on which their treatment started using GD as determined by counting backward from birth date, GD as estimated by the regression of the greatest fetal length obtained ultrasonically, GD estimated behaviorally, or the average of these three estimates ($P > 0.10$ for all). Nor were the dosages of flutamide given to the pregnant mothers, which varied according to maternal weight, significantly correlated with the males' genital masculinization scores, although this measure produced by far the largest correlation ($P > 0.10$). The relationship between daily maternal T and the genital masculinization scores could be determined only for cohort 2 males and was not significantly correlated on any single day T was assessed ($P > 0.10$ for all). Although it is not possible to correlate dose of flutamide or maternal T with the effects of flutamide treatment, it is of interest that the mother of the EFM that had normal genitalia (a score of 16) also

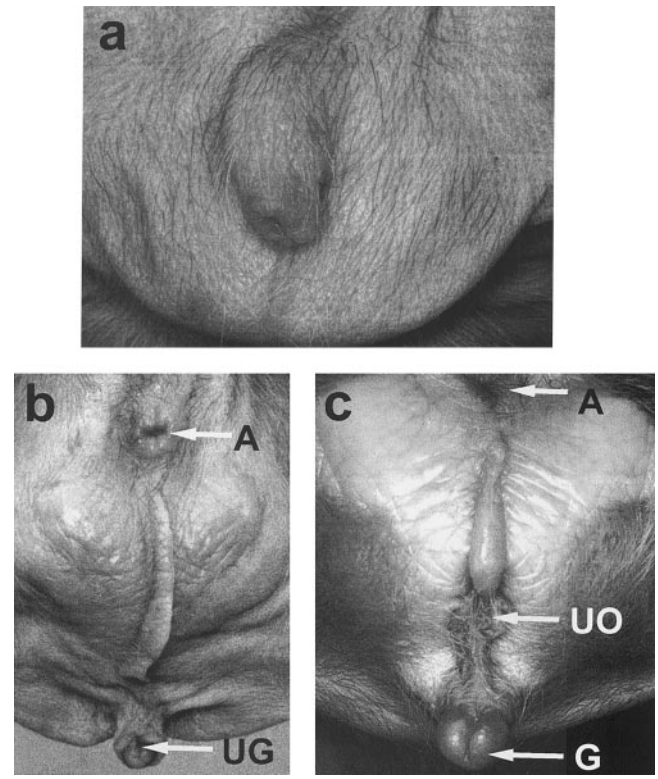


FIG. 3. Three EFMs showing variation in response to treatment. (a) Frontal view of subject RJs5 at 2 months of age. RJs5 has the least altered genitalia of the seven EFMs, with normal arrangement of the phallus rostral to the scrotum. The phallus is smaller than average. (b) Rear view (anus (A) at top) of subject RBh6 at 2 months of age, illustrating the most common variation. RBh6 has a prominent midline raphé extending from the anus to the small, caudal phallus. The scrotum is also small, and a urethral groove (UG) is present at the urethral opening. (c). Rear view (anus (A) at top) of RVu5 at 2 months of age, who is the most altered EFM. His scrotum is rostral and poorly formed, and he lacks a true phallus. A prominent midline raphé extends from the anus to the urethral opening (UO), which is separate from the urethral groove on the glans (G).

had the highest prenatal T levels of any flutamide-treated mother and, because of her small size, was given the lowest daily doses of flutamide during treatment compared with the mothers of other EFMs. In addition, this male was the only EFM to come from the highest ranking matriline.

Neonatal Endocrine Function

All subject groups showed elevated serum LH during the first months of life, with the peak levels at 1 month of age, and then falling to undetectable levels by 6 months in most subjects. In both females and males, LH levels did not change significantly from 3 months to 18 months of age (*post hoc* Tukey test, $P > 0.10$ for all). Over the first 3 months, LH levels in control males were significantly higher than those in control females (2×2 ANOVA, $F_{1,47} = 4.58$, $P = 0.037$; data not shown), but the difference was significant only at 1 month (simple comparisons, $P = 0.001$). Treatment groups of females differed significantly in serum LH over the first 18 months (2×2 ANOVA, $F_{4,268} = 4.61$, $P = 0.001$; Fig. 4a), and EAFs had significantly higher LH than all other female groups except EFFs (*post hoc* Tukey test; LAF, $P = 0.024$; LFF, $P = 0.002$; VCF, $P = 0.003$). The difference between EAFs and VCFs was significant at 1 month ($P < 0.001$). Treatment groups of males, however, did not differ significantly in serum LH over the first 18 months (2×2 ANOVA, $F_{4,244} = 2.18$, $P = 0.072$; Fig. 4b). Serum T levels in males also were elevated neonatally and roughly followed LH changes; levels were highest in the first month and did not change significantly after 2 months (*post hoc* Tukey test, $P > 0.10$). Treatment group affected serum T levels (2×2 ANOVA, $F_{4,243} = 2.56$, $P = 0.039$; Fig. 4c), and LFMs had higher T than all other male groups except LAMs (*post hoc* Tukey test; EAM, $P = 0.011$; EFM, $P = 0.011$; VCM, $P = 0.042$). The difference between LFMs and VCMs was significant at 2 weeks ($P < 0.001$), but not at any later age.

At 8 or 9 and 18 months of age, no subject responded to the GnRH challenge with a greater than 50% increase in LH with the exception of two females at 18 months (one VCF and one EFF). At 2 months of age, control males showed a significantly greater percentage change in LH than did control females (one-way ANOVA, $F_{1,12} = 6.26$, $P = 0.028$), with only three of seven control females responding (greater than 50% increase in LH) compared to six of seven control males responding (Fig. 5). Treatment did not affect the percentage change of LH at 2 months when all female subjects or all male subjects ($P > 0.10$, Fig. 5) were compared. However, not a single EAF ($n = 6$) responded to the challenge with a greater than 50% increase in LH while two of seven LAFs, four of seven EFFs, two of seven LFFs, and three of seven VCFs did respond. The percentage change in T for the males also showed no effect of treatment ($P > 0.10$; data not shown). Similar results were obtained when the magnitude of the change in LH and T in response to the challenge was analyzed.

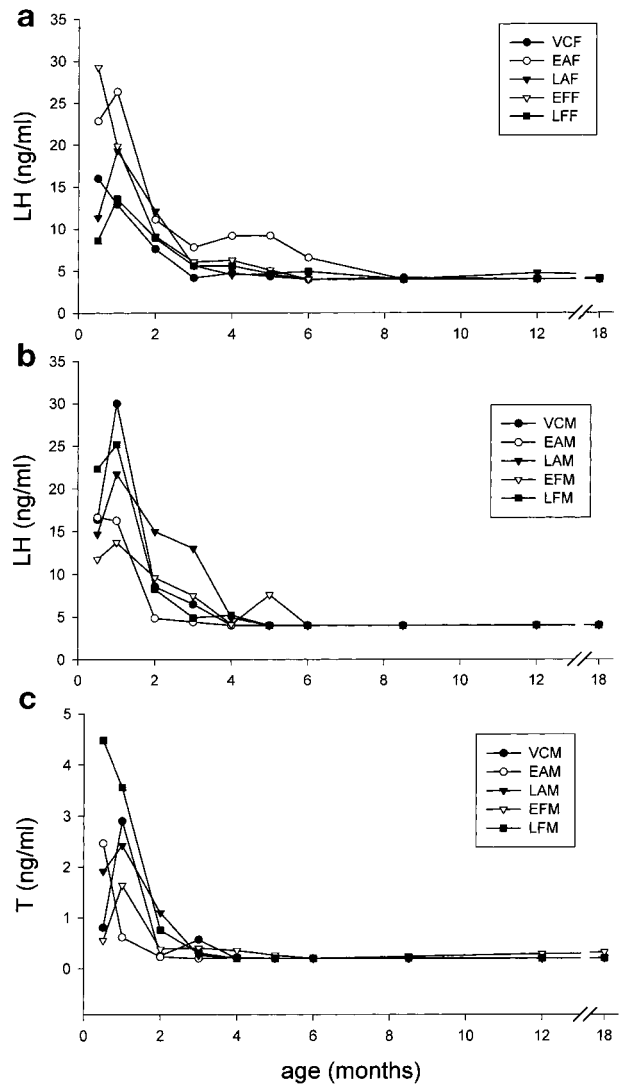


FIG. 4. Mean serum levels over the first 18 months of life by treatment group of (a) LH in rhesus monkey females, (b) LH in rhesus monkey males, and (c) T in rhesus monkey males. Abbreviations as in Fig. 2.

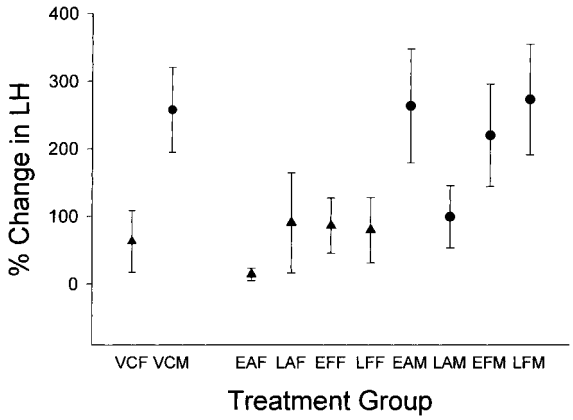


FIG. 5. Mean ± SE percentage change in LH 60 min after an IV injection of GnRH (50 ng/kg BW) in 2-month-old subjects. (▲, females; ●, males). Abbreviations as in Fig. 2.

DISCUSSION

This study is the first to alter sexual differentiation by blocking the actions of endogenous prenatal testosterone in rhesus monkeys. Alterations in the prenatal androgen environment of both male and female rhesus monkeys affected genital differentiation, endocrine function, and growth of rhesus monkey infants. Flutamide treatment, when given early in gestation, produced marked abnormalities in male genital differentiation, producing genitalia neither male nor female in form, but with characteristics of both sexes. Flutamide treatment later in gestation decreased male penis size, but did not alter the basic pattern of male genital organization. Penis length in males treated with TE late in gestation tended to be larger than that in controls, but not significantly so. Since neonatal T (Wallen *et al.*, 1995) and pubertal T also increase penis size, it seems likely that phallic tissue does not have a period of maximal sensitivity, but that the end organ remains sensitive to androgen's actions, at least through puberty. In females, flutamide treatment early in gestation altered genital development, producing smaller AV/AC ratios. Larger AV/AC ratios have been suggested in humans to indicate elevated prenatal androgen exposure (Callegari *et al.*, 1987). Thus, this effect of early, but not late, flutamide suggests that female rhesus fetuses may be naturally exposed to meaningful levels of endogenous androgen. Surprisingly, prenatal androgen treatment of females affected genital anatomy similarly to early flutamide treatment. This contrasts previous studies using much higher androgen doses (Goy, 1981; Goy *et al.*, 1988) in which early but not late androgen treatment extensively masculinized the female genitalia. This finding raises several possibilities, among them the presence of prenatal feedback mechanisms that are activated by low levels of androgen such that total fetal exposure to androgen is reduced.

The effects of early flutamide treatment on genital differentiation in males were highly variable, ranging from fully masculinized, but small male genitalia to frankly intersex genitals. Variation to this extent was not observed in any other treatment group and may stem from the relatively low affinity of flutamide for the androgen receptor (Edelson, 1984; Simard, Luthy, Guay, Belanger, and Labrie, 1986), making it difficult to maintain a complete occupation of androgen receptors with flutamide. This is particularly problematic because blockade of the receptor with flutamide is also likely to remove negative feedback control of gonadotropin secretion (Veldhuis, Urban, and Dufau, 1992; Kerrigan, Veldhuis, and Rogol, 1994), increasing fetal T secretion. Under such conditions, any momentary reduction in the flutamide androgen receptor blockade would allow the larger pool of fetal T to interact with androgen receptors. Thus the variation in the effects of flutamide on male genital development may reflect individual differences in how flutamide is metabolized and the completeness of the blockade. Similar variability has been reported in humans exposed to the androgenic drug norethindrone during fetal life (Jacobson, 1962). Interestingly, the genitalia of our EFMs are similar to those obtained from females exposed to the more rapidly metabolized androgen, dihydrotestosterone (Goy, 1981).

Prenatal androgen manipulation also altered neonatal endocrine function. While no treatment effects on serum LH levels in neonatal males were detected, LFMs showed higher levels of T neonatally than controls. This increase, without a corresponding significant increase in LH, likely indicates an effect on testicular sensitivity to LH, either directly from blocking androgen prenatally or as a result of the flutamide itself. In females, EAFs, but not LAFs, exhibited a greater secretion of LH neonatally than did controls, a finding that corresponds to previous studies of the effects of prenatal androgenization on adult female rhesus monkeys (Abbott *et al.*, 1997; Dumesic, Abbott, Eisner, and Goy, 1997). Abbott and co-workers found that females exposed to high levels of androgen at various times and durations prenatally were more likely to exhibit hypersecretion of both LH and androgen and oligo-ovulation as adults than controls (Abbott *et al.*, 1997). These same females, if given andro-

gen early in gestation, also experienced delayed menarche, but showed normal menarche if treated later in gestation (Goy, Uno, and Sholl, 1989). Human females with congenital adrenal hyperplasia are also more likely to show ovarian hyperandrogenism and LH hypersecretion beginning at puberty (Barnes, Rosenfield, Ehrmann, Cara, Cuttler, Levitsky, and Rosenthal, 1994). Thus, the limited effects seen in infants may become more pronounced at puberty. Males at 2 months of age were more responsive to GnRH injection than were females, but prenatal treatment produced no consistent effects on infant response to GnRH in either sex. However, the finding that only EAFs did not have a single subject who produced a more than 50% increase in LH in response to GnRH may be significant as the animals enter puberty.

There were no significant sex differences in somatic growth, and prenatal androgen manipulations had surprisingly small effects on growth during the first 18 months of life. However, control males tended to be larger than control females on all morphometric measurements. Late androgen treatment increased general body size in males, but early androgen treatment had little effect on somatic growth in either sex. In contrast, early flutamide treatment decreased body size in females, but not in males. In males, the higher CR/CH ratio of the EFMs indicates that early flutamide treatment altered body proportions such that the limb/trunk ratios were smaller in these animals, although overall body size was not affected.

Androgen and flutamide given to pregnant females reaches the fetal circulation, but at lower levels than in the maternal circulation. When pregnant ewes received a single 100-mg injection of testosterone cypionate (comparable on a per kilogram basis to our 20-mg TE injection), T levels in fetal lambs increased significantly in the week following the injection (Wood, Ebling, I'Anson, Bucholtz, Yellon, and Foster, 1991). Slow-release T pellets implanted in pregnant ferrets on day 30 of the 42-day gestation period produced a 150- to 350-fold increase in maternal plasma T but only a 2- to 5-fold increase in the fetal plasma T (Tobet, Shim, Osiecki, Baum, and Canick, 1985). Treatment of pregnant rhesus monkeys with 25-mg TP injections daily for 10 days beginning on GD 40 produced maternal T levels above 125 ng/ml, whereas fetal levels averaged around 5 ng/ml. Although these authors detected no statistically significant increase in fetal T in the umbilical artery at the end of the treatment (Resko, Buhl, and Phoenix, 1987), this same TP dosage produces extensive genital masculinization of fetal females (Goy *et al.*, 1988). Our androgen-treated mothers had clearly elevated T levels during pregnancy, but these were only one-sixth of that reported by Resko *et al.* (1987). Thus, our treatments likely produced fetal levels of 1 ng/ml or less in females. The extent of the androgen block induced by flutamide in the fetus is undetermined. However, without knowing the exact degree of exposure to either compound, we have detected significant alterations in early morphological development that may presage later pubertal and adult effects.

Genital anatomy has been historically the sole determinant of a human's sex of rearing. In addition, human behavioral sex differences are often attributed to a cascade of differential treatment from fellow humans that start with the perception of the structure of the genitalia. Money and Ehrhardt (1972) posit that an individual's gender identity is completely malleable at birth. This assumption becomes an issue when children are born with ambiguous or malformed genitalia or severely injure their genitalia at an early age. Because of the assumed importance of genital anatomy for gender identity, young boys with penises that will not function normally sexually as adults are routinely castrated, have a vulva and vagina constructed, and are raised as females. Recent evidence suggests that a sexually functioning penis of average size is not required for male gender identity (van Wyk and Calikoglu, 1999), and gender identity and behavioral sex differences in humans may not depend solely on treatment by family and peers, but are biased prenatally (Diamond and Sigmundson 1997). van Wyk and Calikoglu (1999) suggest that differences in the success of gender reassignment of males born with micropenis may depend upon the degree of androgen exposure the fetal brain received.

Nonhuman primate data suggest that behavioral sex differences do not depend upon the anatomy of the genitalia, but reflect alterations in the neural systems subserving behavior that are shaped by prenatal hormonal events (Goy *et al.*, 1988). Previous studies have used androgen treatments that produced extensive genital masculinization alterations unlike those employed here. While the effects of massive doses of androgen may be of theoretical interest, they are unlikely to reflect variations in androgen that are likely to occur naturally. It is alterations in androgens that leave little or no external indication of their presence that may be more germane to understanding the role androgens play in shaping gendered behavior (Udry, Morris, and Kovenock, 1995). Our treatments produced, in many cases, subtle or nondetectable somatic effects, although there was evidence that meaningful

levels of active compounds reached the fetus. The long-term effects of such less pronounced prenatal manipulations on behavior and reproductive function are of particular interest.

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