SEX HORMONES, PREGNANCY, AND THE IMMUNE RESPONSE

THE MECHANISM OF PHENOTYPIC SEX DIFFERENTIATION

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For the first 2 months of gestation, there is no difference in the development of male and female embryos. Gonadal differentiation then ensues, and the ovary and testis commence to synthesize their characteristic hormones at approximately the same time. The initial secretion of testosterone by the fetal testis and 17β-estradiol by the fetal ovary appears to be independent of gonadotropin control. If an ovary or no gonad is present, the resulting phenotype is female; thus no gonadal hormones appear to be required for female development. In contrast, two secretions of the fetal testis are responsible for the imposition of the male phenotype. Mullerian regression factor, a poorly characterized peptide hormone, is responsible for suppression of the mullerian ducts, and testosterone accounts for the remainder of male development. The mechanisms by which testosterone causes this differentiative process have been deduced from studies in humans and animals of single gene mutations that interfere with androgen action. Testosterone itself is responsible for virilization of the wolffian ducts into the epididymis, vas deferens, and seminal vesicles, whereas the testosterone metabolite dihydrotestosterone induces the development of the male urethra and external genitalia. Both testosterone and dihydrotestosterone act by combining in the cell cytosol with a high affinity receptor protein that is translocated to the cell nucleus. In summary, chromosomal sex determines gonadal sex, and gonadal sex in turn dictates phenotypic sex.

According to the concepts formulated by Jost (1), sexual differentiation can be viewed as a sequential process beginning at the moment of conception when chromosomal sex is established (Figure 1). However, in human beings male and female embryos develop in an identical fashion for approximately the first 2 months of fetal life. The gonads then differentiate into ovaries or testes, and as a consequence of this gonadal differentiation the male and female sexual phenotypes develop. Presentations by Drs. Wachtel and Ohno in this volume summarize the current concepts of how chromosomal sex is translated into gonadal sex (pages 1200 ad 1211). The purpose of this discussion is to describe the current working model of the mechanisms by which gonadal sex is translated into phenotypic sex. Whatever the differentiative events that cause an indifferent gonad to develop into a testis or an ovary, it is through the action of the gonads as endocrine organs that phenotypic sex develops. Two aspects of the process will be reviewed: 1) characterization of the onset of endocrine function in the fetal gonads and 2) a summary of the evidence of how androgens act in the formation of the male phenotype.

ONSET OF ENDOCRINE FUNCTION IN THE FETAL GONADS

A time sequence study of estradiol and testosterone synthesis in the gonads of the rabbit embryo from day 16 to day 19 of gestation is summarized in Figure 2 (2). The study was designed to encompass the period of

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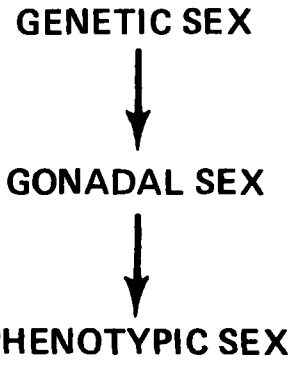

Figure 1. The Jost formulation of the sequence of events in sexual differentiation.

gonadal and phenotypic sexual differentiations in the rabbit; histologic differentiation of the testis begins on day 16, and phenotypic sex differentiation begins between days 17 and 18. During a 12-hour period between days 17 and 17.5 of gestation, estrogen synthesis commences in the fetal ovaries but not in the fetal testes, and testosterone synthesis, expressed as 3β-hydroxysteroid dehydrogenase activity which is rate limiting for testosterone synthesis in this species, commences in the

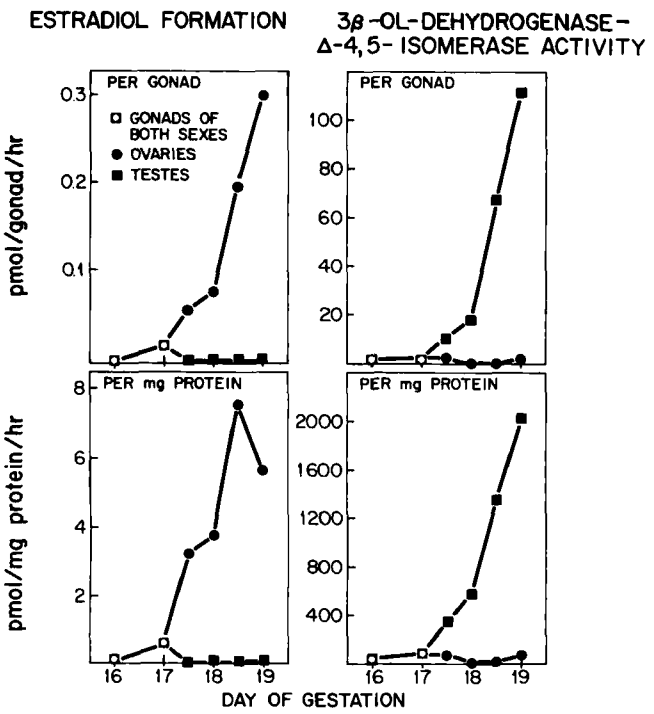

Figure 2. Estradiol formation and 3β-hydroxysteroid dehydrogenase activity (testosterone synthesis) in gonads of rabbit embryos from 16–19 days gestation (2).

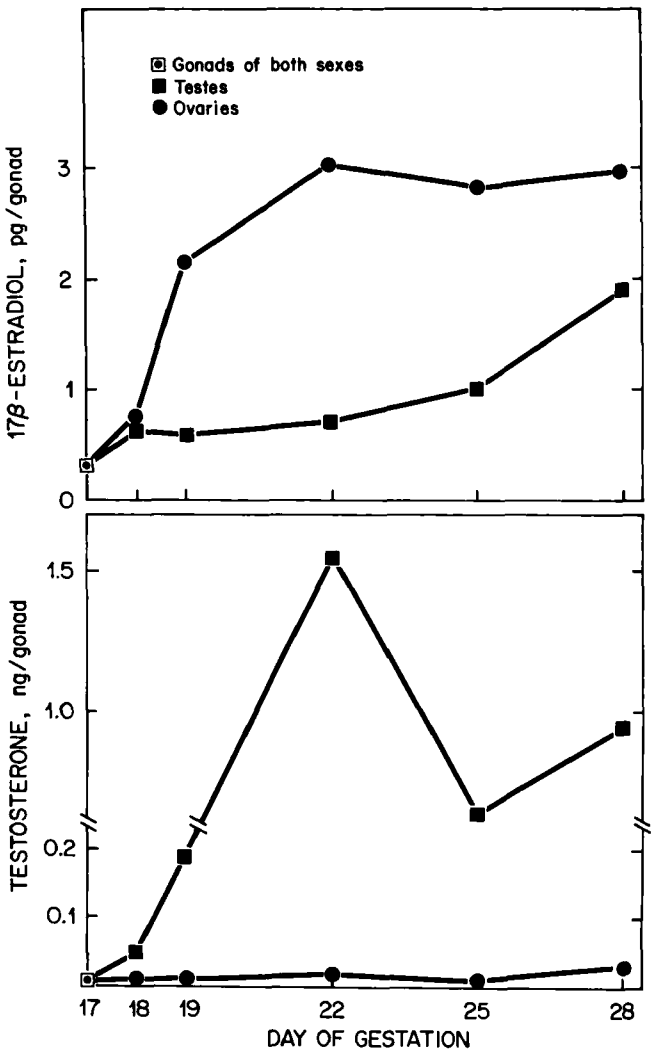

Figure 3. Estradiol and testosterone content of fetal rabbit gonads (3).

testes under circumstances in which the activity in the fetal ovaries is low. Thus, despite the fact that histologic differentiation of the ovaries is almost undetectable at this time, the characteristic endocrine function of the gonads of the two sexes develops at virtually the same time in embryonic development. As demonstrated in Figure 3 this differentiation in endocrine function is not limited to enzymatic development but is also reflected by increase in the content of estradiol in the ovaries and testosterone in the testes (3). Furthermore, the simultaneous onset of endocrine function in the testes and ovaries is not limited to the fetal rabbit but can also be demonstrated in human fetal gonads, in which estrogen synthesis in the ovaries and testosterone synthesis in the

testes both commerce between 6 and 8 weeks of gestation (4).

The enzymatic profile of the pathway of steroid hormone biosynthesis in the fetal rabbit gonads is summarized in Figure 4 (5). On day 18 of gestation only two enzymatic differences can be detected in the steroid hormone-synthesizing machinery between the two sexes: 1) there is about 100 times as much 3β-hydroxysteroid dehydrogenase in the testis as in the ovary so that more testosterone is synthesized in the testis, and 2) the fetal ovary has the capacity to convert the small amount of testosterone synthesized in the ovary into estradiol whereas the testis does not. In summary, the only enzymatic differences between the gonads of the two sexes in the embryonic rabbit are that the testis has more 3β-hydroxysteroid dehydrogenase activity than the ovary and that the ovary, but not the testis, has the capacity to convert testosterone to estradiol.

Two additional points about this pathway deserve emphasis. First, the initial events in steroid hormone synthesis are quantitatively similar in the two gonads; that is, the amount of cholesterol side-chain cleavage activity that converts cholesterol to pregnenolone and the enzymatic capacity to convert pregnenolone to androstenediol are similar in ovary and testis. It is generally believed that the step in steroid hormone synthesis that is regulated by gonadotropic hormones is the side-chain cleavage reaction by which cholesterol is converted to pregnenolone. However, at this stage of development, cholesterol side-chain cleavage activity appears to be gonadotropin-independent in both the ovary and testis until after phenotypic sexual differentiation is well advanced. Indeed, since gonadotropin dependence of testosterone synthesis does not develop until shortly before delivery in the rabbit and since the rate-limiting enzymatic process for testosterone synthesis also develops in cultured fetal testes (6), we conclude that fetal pituitary hormones are probably not involved in the initiation of steroid hormone synthesis in the fetal gonads. The precise mechanism by which steroid hormone synthesis is regulated prior to the onset of gonadotropin control is still not clear. Second, although there are species differences in the development of the enzymatic pathways for testosterone synthesis in the fetal testis, the onset of testosterone synthesis occurs just before the onset of male phenotypic development in all species studied.

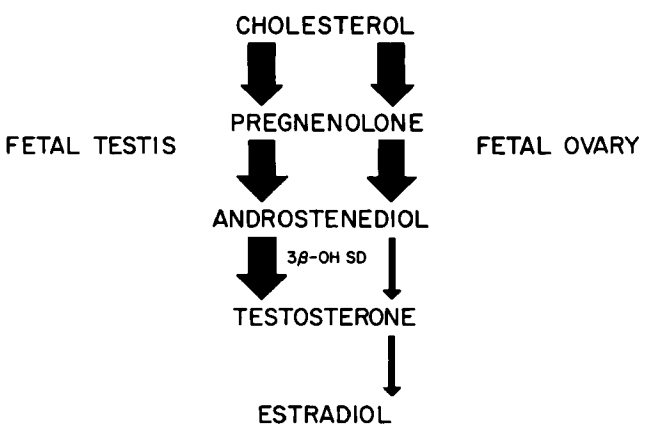

Figure 4. Enzymatic profile of steroid hormone biosynthesis in the rabbit embryo at day 18.

To summarize, histologic differentiation of the testis is followed very shortly by the onset of the endocrine function of the tissue, as a result of which testosterone synthesis is activated. At the same time in the ovary, before histologic differentiation is apparent, estrogen synthesis is activated. Both of these processes appear to be initially independent of gonadotropin control.

PHENOTYPIC DIFFERENTIATION OF THE EMBRYO

An understanding of the endocrinology of the fetal ovary and fetal testis is essential for understanding the subsequent events in phenotypic differentiation. The process of phenotypic sexual development is summarized schematically in Figures 5 and 6.

Prior to the onset of endocrine function of the gonad (at the end of the second month in the human embryo and at approximately 17 days gestation in the rabbit embryo), the internal and external genitalia are identical in both sexes. Adjacent to the gonad is the mesonephric kidney system, consisting of a mesonephros proper and a mesonephric or wolffian duct that terminates in the sexually indifferent urogenital sinus. During the few days preceding the onset of phenotypic differentiation, a second or mullerian duct system has separated from the wolffian duct and also terminates in the urogenital sinus. If the gonad develops into an ovary, the mesonephric kidney system atrophies, and the mullerian system persists; the upper portion of the mullerian duct becomes the fallopian tube, and the fused portion becomes the uterus and contributes to the embryogenesis of the vagina. If the gonad develops into a testis, the opposite happens; namely, the mesonephric system persists, and the mullerian ducts regress. The

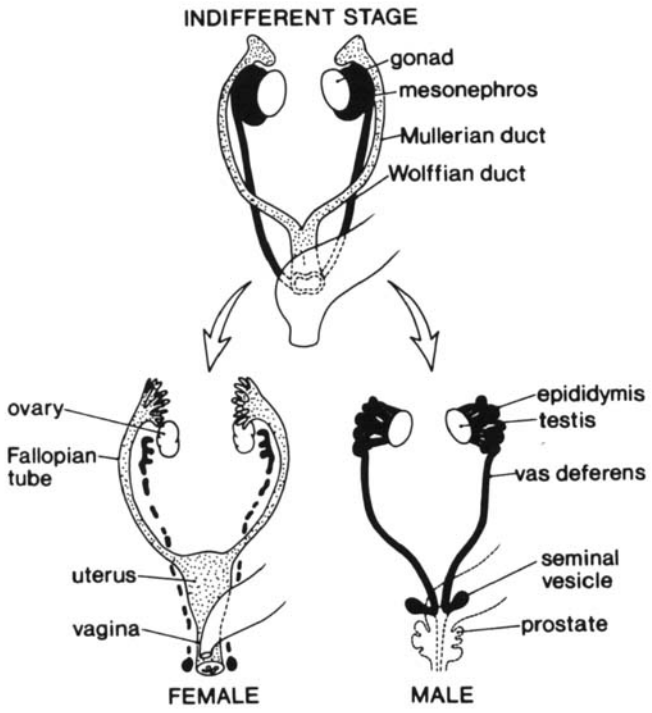

Figure 5. Diagrammatic representation of the differentiation of the internal genitalia.

mesonephros proper (the old mesonephric kidney) becomes the epididymis, the mesonephric duct becomes the vas deferens, and the seminal vesicle differentiates at the end of the mesonephric duct. In addition, there is virilization of the urogenital sinus into the upper portion of the male urethra and the prostate. Thus, the internal accessory organs of reproduction in the two sexes develop from different anlage, the wolffian or mesonephric duct system in the male and the mullerian duct system in the female.

In contrast, the external genitalia in the two sexes develop from common anlage. At the same time that the internal genitalia are sexually indifferent, the external genitalia are also sexually indifferent and consist of a genital tubercle, a genital fold and groove into which the sexually indifferent urogenital sinus empties to the outside, and a genital swelling on each side of the genital fold. If the gonad develops into an ovary, this system changes little except to elongate. The genital tubercle becomes the clitoris. The urethral fold and groove becomes the labia minora, and the genital swellings becomes the labia majora. However, if the gonad develops into a testis, there is fusion of the urethral fold and groove, starting posteriorally and moving anteriorally, ultimately bringing the urethral orifice to the end of the genital tubercle. By this fusion process, the genital folds become converted into the body of the penis. The fusion of the genital folds also transforms the two genital swellings into one structure, the scrotum, which will serve as the receptacle for the descent of the testes.

The evidence that these events, namely the differentiation of both the internal and the external genitalia, are dictated by gonadal sex came from the experiments of Jost in which rabbit embryos were castrated on day 17, prior to the onset of phenotypic sex differentiation (1). Regardless of whether the embryo was a genetic male or female, removal of the gonads prior to the onset of phenotypic differentiation resulted in the development of a female phenotype. As a consequence, Jost concluded that the imposition of the male phenotype upon the sexually indifferent embryo is determined by

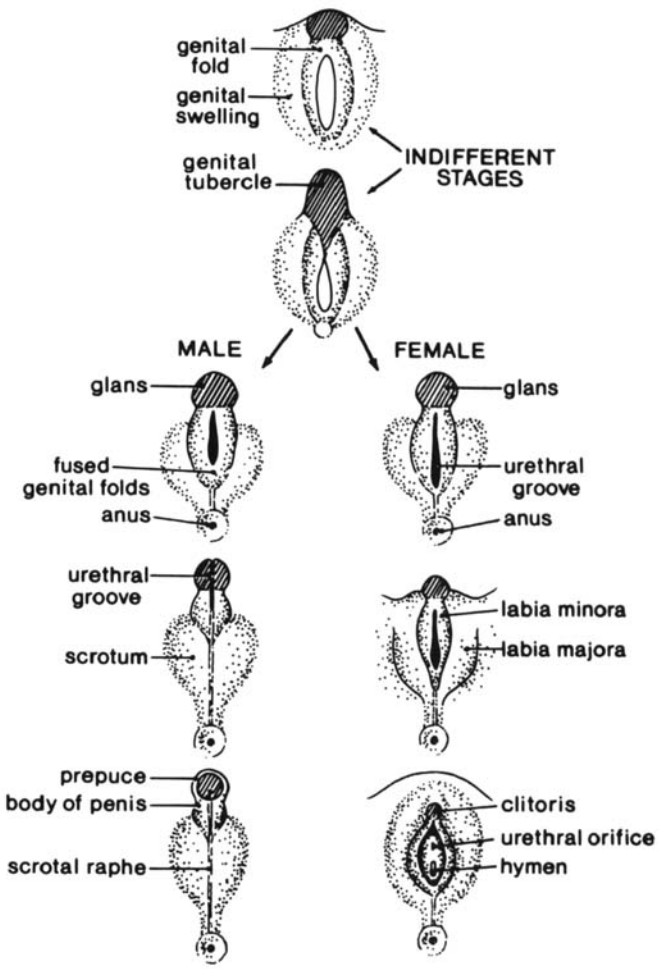

Figure 6. Diagrammatic representation of the differentiation of the external genitalia.

secretions from the fetal testis and that no secretion from the fetal ovary is necessary for a female phenotype to develop. The validity of these conclusions has been confirmed by a variety of studies.

As a result of work done in several laboratories, it has been established that three hormones from the fetal testis are responsible for the imposition of the male phenotype (7). Two of the hormones are secreted directly by the fetal testis itself. The first, mullerian regression factor, an incompletely characterized protein, is responsible for the regression of the mullerian ducts. The second hormone secreted by the testis is testosterone, which is responsible for a major portion of male phenotypic differentiation. In other instances, testosterone from the testis is converted in target tissues to dihydrotestosterone, which is responsible for the remainder of male differentiation.

Despite the fact that the importance of these three hormones in male development was recognized several years ago, there was a delay in analyzing how the hormones act within cells to cause differentiation. For technical reasons, the attempt to characterize directly the receptor machinery for hormone action in embryonic tissue has not been successful because of the small amounts of tissue available. Consequently, most of what we know about how these hormones act within the cell has been derived from studies of single gene mutations in humans and animals, which cause both resistance to the action of androgen and abnormal sexual development. The current views on how androgens act inside target tissue cells to cause virilization are summarized in Figure 7.

Testosterone, the major hormone secreted by both fetal and adult testes, enters the cell by a passive diffusion process down an activity gradient. In the cell testosterone can undergo one of two fates. It can combine directly with a receptor protein, or it can be reduced to dihydrotestosterone. Dihydrotestosterone in turn combines with the receptor. The bulk of evidence suggests that there is one receptor protein that mediates the action of these two hormones. The hormone-receptor complexes then diffuse or are translocated into the nucleus, where they are thought to attach to specific binding sites on the chromosomes and as a result of still uncertain mechanisms promote the transcription of new messenger RNA. Ultimately new messenger RNA reaches the cytoplasm of the cell, and new proteins are synthesized. The testosterone-receptor complex is responsible for regulating secretion of luteinizing hormone by the hypothalamic-pituitary system and for virilization of the wolffian ducts during embryogenesis. The dihydrotestosterone-receptor complex is essential for virilization of the external genitalia of the male during embryogenesis and also for sexual maturation at the time of male puberty. It is uncertain whether testosterone or dihydrotestosterone is responsible for the androgen-mediated events in spermatogenesis.

HEREDITARY DEFECTS IN ANDROGEN ACTION

Proof that testosterone is required for wolffian duct stimulation, that dihydrotestosterone is required for external virilization, and that the androgen receptor mechanism is essential for the action of both hormones during embryogenesis has come from studies of single gene mutations that affect this pathway (8,9). Three different types of mutations have proved to be useful in

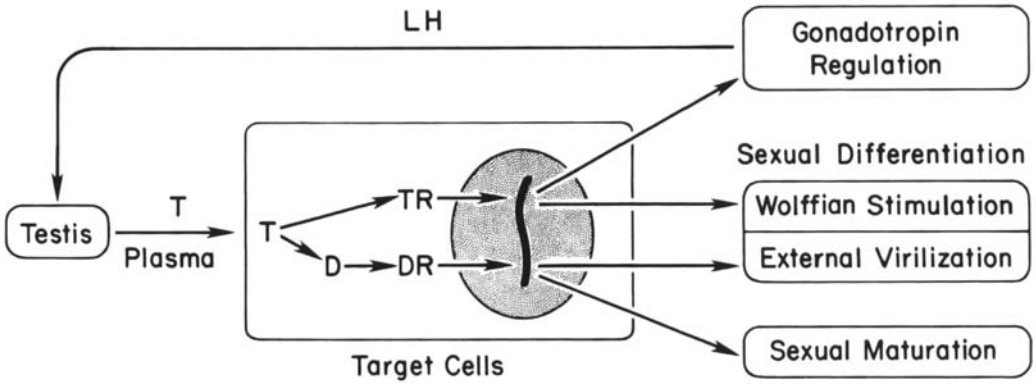

Figure 7. Schematic diagram of the mechanisms by which testosterone accomplishes its major functions. **T**, testosterone; **D**, dihydrotestosterone; **R**, receptor protein.

this regard (Figure 7), namely mutations in the 5α-reductase enzyme that converts testosterone to dihydrotestosterone, mutations in the cytoplasmic receptor protein that is responsible for the binding of androgen and its translocation into the nucleus, and mutations that cause so called post-receptor resistance in which the 5α-reductase enzyme and the receptor appear to be normal but there is some defect in one or more of the steps in androgen action inside the nucleus.

5α-reductase deficiency is an autosomal recessive disorder in which 46,XY males with bilateral testes have normal plasma gonadotropins, normal male testosterone production and plasma levels, and normal virilization of wolffian ducts but failure of virilization of the external genitalia (10–15). The androgen receptor in this disorder is normal. Affected patients were originally described under the term pseudovaginal perineoscrotal hypospadias, a phenotypic designation that was applied subsequently to some patients with disorders other than 5α-reductase deficiency. Clitoromegaly develops at the time of puberty, but on physical exam of the introitus there is a normal female urethral orifice and (usually) a normal vaginal orifice. When these patients are examined in greater detail as shown diagrammatically in Figure 8, they have testes, normal male epididymides, normal vasa deferentia, normal seminal vesicles, and normal ejaculatory ducts. However, the ejaculatory ducts, instead of terminating in a male urethra, empty into the blind-ending vagina. In summary, testes are present, and anatomic development is that of a normal male down to the urogenital sinus. However, the urogenital sinus and external genitalia are predominantly female rather than male.

On the basis of the work done in embryos (16), we predicted that this anatomic defect would develop if there were an absence of dihydrotestosterone formation. Indeed, the phenotype that would be expected if testosterone-mediated events were normal would be male virilization of the wolffian duct and normal gonadotropin regulation, which are both present in this disorder, but defective external virilization and inadequate development of male secondary sexual characteristics at the time of expected puberty.

With this prediction in mind, we studied biopsy material and cultured fibroblasts from the skin of patients with this disorder (10–13) (Figure 9). In the first patients studied (10–13) there was an almost undetectable 5α-reductase activity in cultured fibroblasts as compared with normal controls. However, it is now clear that there are at least two classes of mutations in this enzyme. In patients with very low enzyme levels there is an abnormality in the enzyme that influences

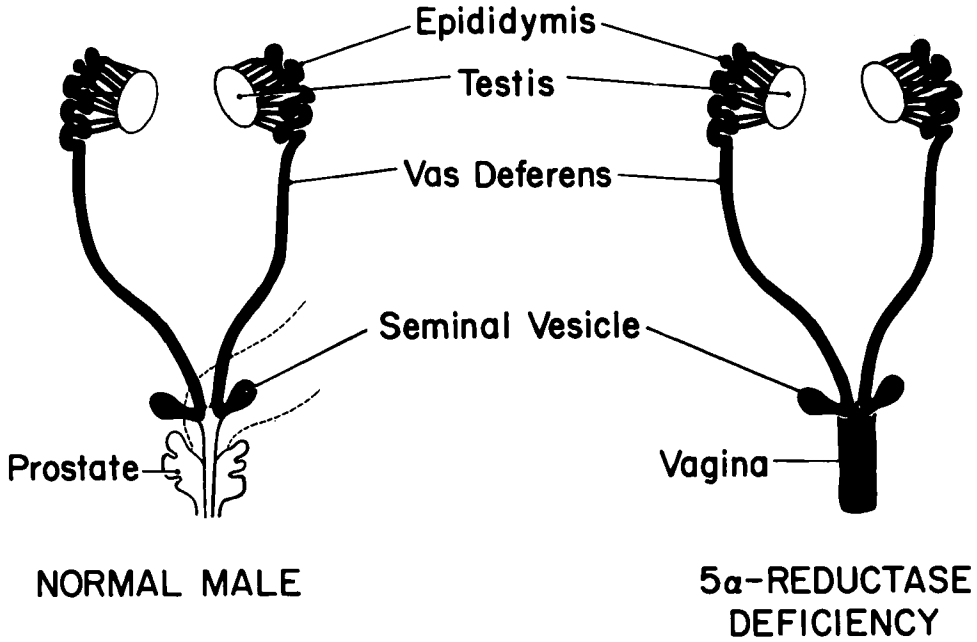

Figure 8. Schematic description of the normal wolffian development and failure of external virilization in 5α-reductase deficiency.

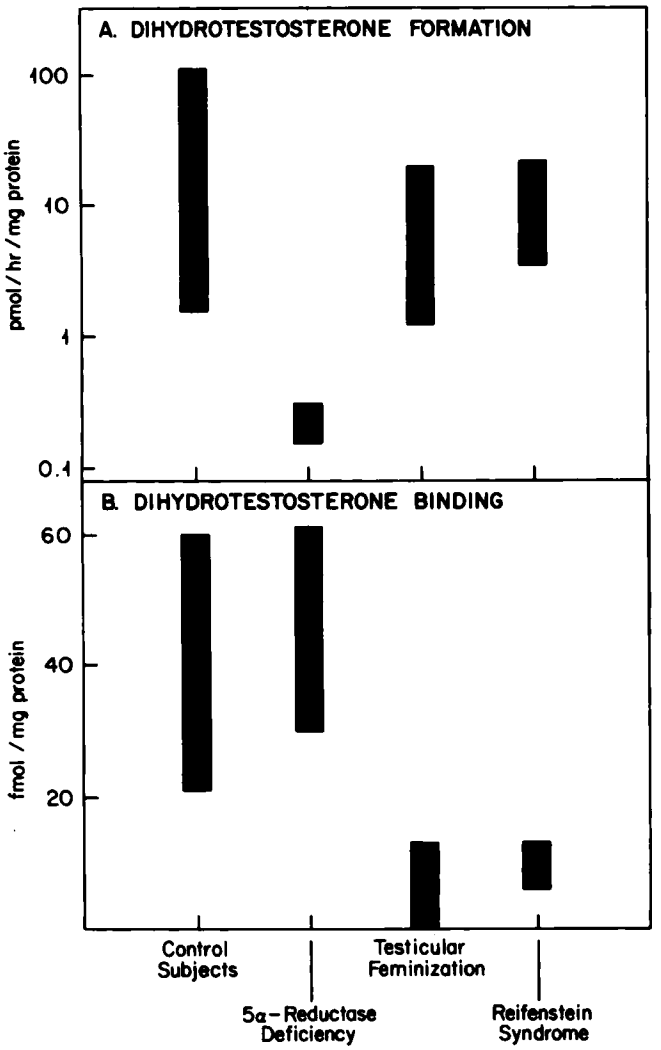

Figure 9. Dihydrotestosterone formation (5α-reductase activity) and dihydrotestosterone binding (androgen receptor) in fibroblasts cultured from the skin of control subjects, patients with α-reductase deficiency, testicular feminization, and Reifenstein syndrome.

the binding sites for the steroid substrate. No patient yet described has a total absence of the enzyme, and the fundamental defect seems to be in the binding of the hormone to the enzyme. The enzyme from some patients, however, binds testosterone normally but has a defect in the binding of the cofactor NADPH (14–15). As a result the 5α-reductase enzyme in the latter group is grossly unstable, and the turnover of the enzyme is exceedingly rapid in vivo.

Thus, at least two types of mutations influence this enzyme and result in a phenotype in which male differentiation of the wolffian ducts is normal but in which the prostate and external genitalia do not virilize. Each affected patient has some detectable dihydrotestosterone in the circulation; this feature of the disorder is the result of deficiency but not total absence of the 5α-reductase enzyme. For example, plasma dihydrotestosterone is detectable at the time of puberty, and this amount of dihydrotestosterone formation may be responsible for the partial virilization that occurs characteristically at the time of expected puberty.

The second class of mutations in androgen action, originally characterized in the mouse by Gehring, Tomkins, and Ohno (17), involves *defects in the androgen receptor.* Although there are several different types of mutations in the androgen receptor in the human and in animals (9), we will focus on the human mutations. Complete testicular feminization in the human is an X-linked recessive trait in which affected 46,XY males have high plasma gonadotropins, high plasma testosterone, and a female phenotype. The mullerian ducts regress, but other aspects of male phenotypic differentiation are abnormal (9). The external genitalia and breast development are those of a normal female, as is the remainder of body development. There is an absence of axillary hair and a paucity of pubic hair, and the testes may either be intraabdominal or in the labia majora. These individuals have blind-ending vaginas and an absence of structures derived from both the wolffian and the mullerian ducts. There is, furthermore, profound resistance both to endogenous androgens and to exogenous testosterone and dihydrotestosterone, even when administered in pharmacologic amounts (8,9).

Thus, the disorder is what would be predicted if there were an abnormality in the receptor that binds testosterone and dihydrotestosterone (Figure 7) so that all the androgen-mediated events are abnormal, including gonadotropin regulation, the events of sexual differentiation that depend on androgen action, and sexual maturation at the time of puberty.

In the mouse mutant and in the first human mutants studied, there was virtually a total absence of the high affinity androgen receptor (18). However, other patients with the phenotype of testicular feminization have detectable but subnormal levels of androgen receptors (Figure 9). Some of these patients have a structural abnormality which results in instability of the receptor (19). Therefore, we believe that the phenotype of complete testicular feminization can result from either absence of androgen receptors or a qualitative abnormality that interferes with receptor function.

Patients with the testicular feminization muta-

tion feminize for two reasons (9). One is that the secretion of estradiol by the testes is increased from about 5 to 70 μg per day. The second factor in the feminization is the androgen resistance itself. Normally there is an antagonism between androgen and estrogens that is not understood in molecular terms, but on empirical grounds such antagonism is known to exist. The administration of 70 μg of estradiol per day to a normal male would result in some feminization but not the florid degree of feminization seen in patients with testicular feminization. Thus, the feminization results from the elevated estrogen secretion plus the androgen resistance so that there is no antagonism by androgen to the action of estrogen.

In addition to mutations that cause testicular feminization by either a qualitatively abnormal or absent receptor, there is another series of mutations in the androgen receptor (8). The most common of these disorders is the Reifenstein syndrome, which is also thought to be X-linked (8,9). The usual expression is a man with gynecomastia and third degree hypospadias (a urethral orifice at the base of the penis rather than at the end of the glans). In contrast to testicular feminization, in which the phenotype is constant within families, in this disorder there is considerable variability in expression (8,9). Some patients have rudimentary vaginas, whereas others have a normal male phenotype except for a bifid scrotum and sterility. Fibroblasts from patients with the Reifenstein syndrome have a deficient but variable amount of androgen receptor. However, in Reifenstein syndrome and in another partial mutation that results in male infertility (20), we have been unable to demonstrate any qualitative defect in the receptor (19,21). The only abnormality that can be identified is a decrease in receptor number. Subtle qualitative defects may be present, but if so, present techniques are inadequate to identify them (19).

In addition to mutations in 5α-reductase and in the androgen receptor, there is a third type of mutation termed *post-receptor resistance* that was described originally by Amrhein and colleagues (22). This is a group of patients with androgen resistance and hereditary male pseudo-hermaphroditism in whom the 5α-reductase enzyme and the androgen receptor appear to be normal. Furthermore, the receptor is translocated into the nucleus in a normal fashion (23), but it has been postulated that there is a defect with the processing machinery inside the nucleus or that there is some subtle defect in the receptor protein that interferes with the function of the hormone-receptor complex in the nucleus. It differs from the previously described disorders in that the amount of androgen receptor is normal and no qualitative abnormality can be detected.

We have studied two individuals from unrelated pedigrees with this disorder (23). These patients had a female phenotype but some clitoromegaly at puberty. Breast development was incomplete. If the abnormality is in the intranuclear processing of the androgen-receptor complex, as postulated, it has to be a generalized defect since all androgen responses seem to be abnormal. That is, plasma gonadotropin levels are elevated, there is failure of male sexual differentiation, and sexual maturation at the time of puberty is deficient.

CONCLUSION

Three classes of mutations in androgen action have been informative for elucidating the events of normal male sexual differentiation: 5α-reductase deficiency, defects in the androgen receptor, and post-receptor resistance. As these various mutations are studied, it has become apparent that there is extreme polymorphism; indeed mutations within each family may be different in character when studied in sufficient detail. Many other mutations in this pathway must exist, and they will be equally valuable in helping us understand normal sexual differentiation as well as provide information on the nature of human defects.

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