The Effects of Diethylstilbestrol (DES) before Birth on the Development of Masculine Behavior in Juvenile Female Rhesus Monkeys

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Eight pregnant rhesus monkeys were injected with 100 μg diethylstilbestrol dipropionate (DESDP) from the 40th day of gestation until term, a long-term treatment. Male (n = 3) and female (n = 5) offspring were obtained. Five other pregnant females were injected with DESDP beginning on the 115th day of gestation and continuing until either the 140th day or term—a relatively short-term treatment. Five female infants were obtained from these short-term treatments. Monkeys from the treated pregnancies were assigned randomly to mother-infant social groups containing untreated male and female infants the same age. They were observed in their peer groups each weekday from 3 to 12 months of age, and the display of mounting and play behavior was recorded for each subject. Results showed that DESDP significantly increased the frequency of display of these juvenile behaviors only in long-term-treated females. However, one of the aspects of mounting that is characteristic of males (the ratio of complete to abortive mounts) was unaffected even by the long-term treatment. Thus, DESDP-treated females displayed a limited behavioral masculinization. Whether this limitation was due to dosage and/or timing or to a selective action of DESDP was not determined. DESDP-treated males were not altered in any measurable way compared to untreated males.

The augmentation of male-like psychosexual characteristics in genotypic females has been accomplished in several laboratory mammals by exposing fetal or larval forms to either natural or synthetic estrogens during early stages of development (rats: Levine and Mullins, 1964; Ladosky, 1963; Whalen and Nadler, 1963; hamsters: Paup, Coniglio, and Clemens, 1972; Whalen and Etgen, 1978; Ruppert and Clemens, 1981; mice: Edwards and Herndon, 1970; gerbils: Ulibarri and Yahr, 1996; guinea pigs: Feder and Goy, 1983; Hines and Goy, 1985). However, no experimental work with nonhuman primates and the behavioral effects of prenatal estrogens has been reported.

In undertaking these studies of the role of estrogen in primate psychosexual development we chose to use the synthetic estrogen diethylstilbestrol rather than the most potent naturally occurring estrogen, estradiol. We did so partly because of the potential value of the work in understanding problems associated with its previous widespread clinical use (Herbst and Bern, 1981), and partly because of its specific biochemical and physiological advantages over the natural estrogens. Relatively small doses of diethylstilbestrol can be used effectively because this nonsteroidal estrogen is not inactivated by binding to blood proteins. Moreover, it has been demonstrated in primates that diethylstilbestrol is degraded much less by the placenta than is estradiol (Slikker, Hill, and Young, 1982). Its previous widespread use in human beings also provided confidence that it would not cause abortion or fetal death when administered over the long period of prenatal development of rhesus monkeys.

In this study, we investigated the effects of exposure to diethylstilbestrol dipropionate (DESDP). No previous work had been done with this substance on development of rhesus psychosexual characteristics, and no prior guidelines existed for when this exposure should occur. We therefore elected to give daily injections throughout most of pregnancy beginning around the start of sexual differentiation and continuing until term (Goy, 1996). We also chose a shorter exposure that coincided with a gestational interval known to be sensitive to the masculinizing actions of testosterone (Goy, Bercovitch, and McBrair, 1988a).
METHODS

Subjects. Adult females from the Primate Center Breeding Colony were time-mated by pairing with adult males of proven fertility. Pairings were carried out when females approached the peak of coloration of the sex skin (usually about Day 10 of the ongoing menstrual cycle). They were left with the selected male continuously until a few days following the abrupt decrease in the intensity of sex skin coloration. Usually the total pairing period lasted about 5 to 6 days, and the day of conception was estimated to be the midpoint of this interval. When each female was estimated to have reached the 40th day of pregnancy, injections of DESDP were begun. Injections were given at 10:00 AM each day.

The long-term treatment began on the 40th day of pregnancy and continued until the infant was born (from 162 to 170 days of gestation, or after about 120 injections of the hormone on average). Five female offspring received this prenatal treatment, and formed a single group referred to as DESlong females. Three male offspring also received this treatment, and are referred to as DESlong males. The other treatment was shorter in duration. It began on the Day 115 of pregnancy. For four females it ended on Day 140, that is, after 25 daily injections, and for one it ended at term. In the analyses that follow, these five females were combined into a single treatment group referred to as DESshort females. This particular interval of treatment was selected because previous work with testosterone propionate had shown that this was a highly effective period for masculinizing the behavior of female fetuses without virilizing their genitalia (Goy et al., 1988a).

All injections were intramuscular, and the injection site was varied daily. The hormone was dissolved in sesame seed oil, and the concentration was 500 μg/ml. Each injection was 0.2 ml (100 μg). Although the total amount injected differed somewhat among subjects given the long-term treatments (because the duration of pregnancy varied naturally), the amount of the difference did not exceed about 800 μg, a small amount compared with the average total of 12,400 μg. We chose 100 μg as a suitable daily dosage based on our successful past experience with DESDP in the masculinization of female guinea pig behavior (Hines and Goy, 1985). In guinea pigs, 3 μg per day was an effective dose, and although adult rhesus weigh only about 14 times as much as adult guinea pigs, we used a dosage about 33 times as large as that used with guinea pigs. Normal males (n = 18) and females (n = 19) were obtained from routine colony breeding in which the pregnant females were not treated. These criterion groups are referred to throughout the text as control males and control females, respectively. Altogether there were five different treatment groups: control males, DESlong males, control females, DESlong females, and DESshort females. These 50 animals were distributed among nine different social groups for rearing and observation.

Housing and observations. When infants were about 6 weeks old, each treated subject and its mother were placed into a large pen (6 feet wide × 7 feet deep × 7 feet high) that also contained two control males and two or three control females and their mothers. The n per study group varied so that all experimental Ss could be accommodated. These study groups formed the social setting for the development and display of juvenile behaviors for the next year. The floor of the "cage" was paved with quarry tile to facilitate cleaning, and drains for waste matter were centered in the floor. The interior of the pen was equipped with stainless steel wire mesh ramps and platforms upon which the animals could rest and/or climb. Stainless steel bars and poles were also installed for climbing and perching.

Animals in each social group of mothers and infants were observed for 30 min each day for 150 successive days beginning when the infants were 3 months old on average. During observation, a focal animal technique was employed whereby each infant subject was observed for 5 min and the display of specified behaviors was recorded on a check sheet. In addition, the occurrence of all mounts during the entire 30-min period was recorded. The reason for this special treatment of mounting is due partly to its special relevance to gender role development, and partly because its rate of display is much lower than that for other behaviors. When the behavior of interest was displayed by a designated subject its occurrence was recorded on a check sheet in such a way as to indicate which animal was the actor and which was the partner. Thus the system of recording permitted determining whether particular responses were shown more often by actors of one sex than the other as well as to partners of one sex more than the other. Preference for either male or female partners is characteristic of young rhesus for specific kinds of behavior (Loy and Loy, 1974). The order in which subjects were observed was rotated daily, and the time of observation was also randomly distributed across the hours from 8:00 AM to 3:30 PM.

Description of behavior. Four behavioral events form the core of data for the present report. These are (1) the display of Complete Mounts, (2) the display of Abortive Mounts, (3) the performance of Rough Play,
and (4) the display of Play Initiating actions and facial expressions. A Complete Mount has the following characteristics: the partner’s leg(s) or ankles are grasped by the actor’s foot or feet, and the actor is oriented correctly at the rear of the partner. The actor nearly always performs pelvic thrusts with this type of mount, but the rare mounts without thrusts are also included. Abortive Mounts were accompanied with pelvic thrusts, but the actor’s feet did not clasp the partner’s legs or ankles. Furthermore the actor could be oriented toward any region of the partner’s body (head, side, rear, etc.). For statistical purposes Complete Mounts and Abortive Mounts were combined into a single measure referred to as Total Mounts. In addition, a Difference Score was calculated by subtracting the number of Abortive from the number of Complete Mounts. This Difference Score equaled zero when the two types of mounts were equal in number. Scores were positive when Complete Mounts outnumbered Abortive Mounts and negative in the reverse case. The Score is sexually dimorphic with positive values more characteristic of males. Rough Play is recognized as a type of wrestling or at least some form of similarly vigorous contact, even if the partner is not thrown to the ground. Play Initiations take the form of grabbing, slapping, grab-and-pull, or a specific facial expression that involves gaping at a partner in which the mouth is open, the lips are slightly retracted, but the teeth remain hidden from view.

**Treatment of data and statistics.** The frequency of occurrence of each of the types of behavior was summed for 50 successive days. Inasmuch as the entire set of observations included 150 days, the sum for each 50 days provided three successive blocks of trials. The data for each subject were divided by the number of possible partners toward which it might have displayed the response (in the present case that number was either 4 or 5). Then the average per partner was summed across all actors of a similar type, and an overall mean and standard error of the mean were calculated for each group of actors. Differences among means were then analyzed for significant differences by multiple factor analysis. The factors evaluated by the anova were Actor Type (i.e., treatment) and Block (which served as a measure of age/development). When the level of significance of the overall $F$ justified it, specific preselected comparisons were made by use of subanalyses of variance so that actors that received DES prenatally could be compared with normal, untreated actors of the same sex. Similarly DES females were tested against normal males, and DES-treated males were compared with normal females. Generally, comparisons between the DES subjects were not carried out. The criterion level for statistical significance was set at $P = 0.05$, and all reported $P$ values are two-tailed. Analyses were carried out on transformed data (square root) with no difference in results, so the analyses reported here are on the nontransformed data.

**RESULTS**

**Play behavior.** The overall differences among groups in the frequency of Play Initiation were highly significant (main effects: for type of actor, $F[4,45] = 5.993, P < 0.001$; for Block, $F[2,90] = 5.304, P = 0.007$; for interaction of actor type X Block, $F[8,90] = 16.852, P = 0.021$). The means obtained for the five groups of actors (i.e., subjects) are shown in Fig. 1. Inspection of the figure shows that males differed markedly from females even during the first Block of observations whereas the other types of actors did not differ from females at that time. During the second and third Blocks, however, differences emerged between DES-long males, DES-long females, and DES-short females on the one hand and control females. Subanalyses showed, however, that the difference between control females and DES-short females was not statistically significant ($F[1,22] = 1.31$, NS) whereas differences between DES-long females or DES-long males and control females were significant ($F[1,22] = 5.783, P = 0.025$; $F[1,20] = 7.564, P = 0.012$, respectively). Thus administration of
estrogens before birth resulted in augmentation of Play
Initiations, but this effect was limited to females and
only apparent when the duration of treatment was
long-term (i.e., from the 40th day of gestation until
birth).

DES10 not only augmented the average frequency of
Play Initiations per partner, but also influenced the type
of partner toward which the initiations were displayed
(Fig. 2). As shown in Fig. 2, there was little difference in
performance of Play Initiation when the type of partner
was female. Under this condition, all means were rela­
tively low (less than 16 occurrences on average in 150
days). In contrast, all groups except control females dis­
payed higher averages than 16 Play Initiations when the
partner addressed was a male. Overall the differences
among means were significant (main effects: for type of
actor, $F(4,45) = 4.816, P < 0.005$; for type of partner,
$F(1,45) = 18.704, P < 0.001$). Moreover, the interaction
between type of actor and type of partner was highly
significant ($F(4,45) = 4.167, P < 0.001$). Inspection of
Fig. 2 shows that the interaction was attributable to the
preferential initiation of play by control males, DES10ng
males, and DES10ng females with male partners com­
pared to the lack of such a preference in DESshort and
control females. Differences in average performance of
Play Initiation with male partners among control males,
DES10ng males, and DES10ng females were not statisti­
cally significant ($F(2,23) = 0.345$, NS).

Analysis of Rough Play yielded results similar to
those shown above for Play Initiation (Fig. 3). The main
difference between these two measures was the uni­
formly higher frequency of performance of Rough Play.
A minor difference was the suggestion in the figure
that differences among types of actors appeared as early
as Block 1. As for Play Initiation, however, the differ­
ences among types of actors in the performance of
Rough Play was very conspicuous in the later Blacks.
Overall, differences were statistically significant (main
effects: for type of actor, $F(4,45) = 21.537, P < 0.001$;
for Block, $F(2,90) = 3.984, P = 0.022$), and the interaction
between Block and type of actor was also significant
($F(8,90) = 2.995, P = 0.005$). The interaction was due to
the fact that DES10ng males were the only treatment
group to show significant increase across blocks in their
performance of this behavior.

Although data are not presented here in order to
conserve space, when the performance of Rough Play
was analyzed for effects of male and female partners,
only control males, DES10ng males, and DES10ng fe­
male showed a preference for playing with male part­
ners (78, 81, and 72%, respectively). Control and DES­
short females showed only about half of all their rough
play to male partners (51 and 55%, respectively).

Mounting behavior. Mean frequencies of Total
Mounts (Fig. 4) differed significantly among groups
(main effects: for type of actor $F(4,45) = 29.501, P < 0.001$;
for Block, $F(2,90) = 3.631, P = 0.03$). The interaction
was not statistically significant. The relevant sub­
analyses demonstrated that DESlong females displayed significantly less mounting than control males ($F_{[1,19]} = 7.564, P = 0.013$) and significantly more mounting than control females ($F_{[1,22]} = 17.506, P < 0.001$), whereas DESshort females closely resembled control females and differed only from control males by showing significantly fewer mounts ($F_{[1,19]} = 19.477, P < 0.001$).

It is of interest to note that the interaction term in these subanalyses was statistically significant when control females were compared either with control males ($F_{[2,70]} = 3.389, P = 0.039$) or with DESlong males ($F_{[2,40]} = 10.905, P < 0.001$), and DESlong males did not differ significantly from control males in this regard. This significant interaction of actor type X Block results from the fact that both control and DESlong males showed large increases in mounting from Block 1 to Block 3 whereas control females did not. Despite an apparently robust sex difference, however, neither group of DES-treated females differed significantly from either the male or female controls. Failure to find any effect of DESDP in this instance may be due to the high variability within the DES-treated groups, because inspection of their means shows that in their constancy across the three Blocks they closely resembled those of the control females (Fig. 4).

Unlike the display of play behavior, no preference for a partner of a particular sex was evident for mounting behavior during the first year of life. Males displayed Total Mounts (Mean and SEM) $10.18 \pm 1.06$ times to females on average, and $9.88 \pm 1.83$ times to males. Females displayed Total Mounts $0.82 \pm 0.15$ and $0.41 \pm 0.11$ times to females and males, respectively (main effect for Sex of Partner; $F_{[1,35]} = 0.047$, NS). Previous studies have confirmed this "indifference" to sex of partner during the first year of life (Deputte and Goy, 1991), and have also demonstrated that marked and predictably changing preferences develop at later ages (Goy, 1996).

A higher proportion of Total Mounts was displayed as Complete Mounts by control and DESlong males than by control, DESlong, or DESshort females (Fig. 5). This inequality of the types of mounting was revealed in the Difference Score obtained by subtracting Abortive from Complete Mounts. As the figure shows, the Mean Difference Scores across all Blocks were consistently positive for control and DESlong males. In contrast, they were nearly consistently negative for control, DESlong, and DESshort females. Two features of this score are especially important to point out. First, the statistically significant effect of Block was attributable to the fact that the mean Difference Scores for control and DESlong males were nearly zero (0.25 and 0.13, respectively) during Block 1, and they increased steadily to 6.24 and 5.80, respectively, in Block 3. Thus during the first few months of life the sexes were equal with regard to the display of the two types of mounts. The second important feature to emphasize is that DESlong females did not show any significant changes in the Mean Difference Scores across the three Blocks despite the fact that they were showing significantly higher
average numbers of mounts than control females. Thus, the DESDP masculinized one aspect of mounting behavior selectively, but DES-treated females failed to show the developmental differential augmentation of Complete Mounts that normally characterizes male rhesus.

The possibility that DESshort females were simply late in the development of masculine characteristics was investigated by restudying them in their social groups at a later age. One of the DESshort females died prior to this later study. Daily observations were carried out between the ages of 24 and 27 months. At that age control males displayed 11.5 ± 3.9 bouts of Rough Play compared to 2.43 ± 0.7 and 3.52 ± 1.94 for control and DESshort females, respectively ($F[2,21] = 3.852, P < 0.038$). However, DESshort females did not differ significantly from control females ($F[1,13] = 0.481$, NS). Results for Total Mounts paralleled those for Rough Play. The overall $F$ for control males, control females, and DESshort females was significant ($P < 0.001$), but the comparison between control females and DESshort females was not ($F[1,13] = 3.744, P = 0.075$).

None of the subjects exposed to DESDP prenatally showed any gross anatomical modifications whatsoever. The clitorides of females were not enlarged or engorged, and the specialized sexual skin of the perineum was not unusually red. The three males produced had normal penises as far as a superficial inspection could discern, and this finding implied that DESDP did not interfere with fetal pituitary gonadotrophin secretion or with testicular androgen production or action.

**DISCUSSION**

Our results for play and protosexual behavior of juvenile rhesus monkeys demonstrate a masculinizing influence of diethylstilbestrol on females exposed to the nonsteroidal estrogen prior to birth. We found such a relationship only for exposure which lasted from the 40th day of gestation until birth. Although play was masculinized by this prolonged exposure (about 125 days duration), the treatment failed to induce mounting that quantitatively equaled that shown by normal males. In addition, Complete Mounts and Abortive Mounts were displayed in nearly equal numbers, a characteristic that is decidedly feminine. In contrast, results obtained when testosterone was administered for only about 80 days of pregnancy produced behavioral changes closely resembling corresponding traits in the genotypic male (compare present mounting and play results with those from previous reports using testosterone; Goy, 1996).

It is quite possible that the differential performance of Complete and Abortive Mounts is experientially determined and that hormones prior to birth only indirectly influence the behavior by determining the type of experience. For example, it could be that the type of hormone before birth is responsible for the growth of a penis. The presence of this organ, in turn, directs the type of mounting that will become characteristic of the individual by providing a sensory feedback derived from appropriate versus inappropriate orientation to the "target." For this mechanism to work intromissions would have to be unneccessary to this sensory feedback, because they do not normally appear in the behavioral repertoire until puberty. An alternative, or added, environmental influence could be the response of the partner to an attempted mount. Partners may be more likely to stand quietly and hold the presentation posture for a phenotypic male than for a phenotypic female. An influence from these factors cannot be ruled out in the present study.

The single shorter exposure that we investigated lasted only from the 115th day of gestation until the 140th day—a period of 25 days duration. We expected a measurable masculinizing effect from this treatment since testosterone propionate (TP) administered during this same interval had clear and statistically significant masculinizing effects on the play and mounting shown by females (Goy et al., 1988a). However, neither play nor mounting behavior showed any evidence of masculinization by this prenatal exposure to the drug.

The pattern of results obtained can be interpreted in several ways. It could be that the dose of estrogen given was too low, but this seems unlikely. The dosage was more than 30 times that used effectively during gestation in guinea pigs. Importantly, the guinea pig like the rhesus has a long pregnancy which encompasses most or all of the period of psychosexual differentiation. An alternative hypothesis is that the participation of androgen is more obligatory in primate psychosexual differentiation than it is in lower mammalian models. This is a possibility that cannot now be rejected, and one which is compatible with the pattern of results shown to be characteristic of human beings.

Such a possibility is suggested by the finding that the hypothalamic–pituitary–ovarian axis seems to be uninfluenced by exposure of human females to diethylstilbestrol prior to birth (Barnes, 1979), and this stands in contrast to the regularity with which such influences are observed in nonprimate mammals. Additional support for a limited organizing action of estrogens in these
higher primates arises from studies showing behavioral masculinization of female rhesus fetuses with dihydrotestosterone (Goy, Uno, and Sholl, 1988b), a non-aromatizable androgen, and this finding also is not commonly paralleled in rodents.

When evidence for masculinizing actions of prenatal DES in humans is reported, the effects are far from uniform. A few fairly recent reports note that adult human females exposed prenatally to diethylstilbestrol show a higher incidence of homoerotic preference than their unexposed control females (Ehrhardt, Meyer-Bahlburg, Rosen, Feldman, Veridiano, Zimmerman, and McEwen, 1985), but only about half of the sample studied showed such an effect. In another report some evidence suggested a masculinization of gender-related behavior during early development (Ehrhardt, Meyer-Bahlburg, Rosen, Feldman, Veridiano, Elkin, and McEwen, 1989). Note, however, that a more recent study from the same clinic failed to replicate this masculinizing effect of the hormone on gender-related behavior (Lish, Ehrhardt, Meyer-Bahlburg, Rosen, Gruen, and Veridiano, 1991). Cognitive functions that normally differ between the sexes seem to be unaffected in prenatally exposed DES females (Hines and Shipley, 1984; Berenbaum and Hines, 1992; see also Hines and Collaer, 1993, for an extensive discussion). Thus, the evidence for masculinizing effects of DES on humans is spotty at best, sometimes contradictory, and seemingly positive only for specific behavioral systems, or fragments of such systems. In short, the pattern of results for human beings does not differ greatly from that reported here for rhesus monkeys.

Oddly, and paradoxically perhaps from some viewpoints, one report suggests that cognitive functions in prenatally exposed human males were affected in a manner that could be interpreted as a feminizing influence (Reinisch and Sanders, 1992). The finding by Reinisch and Sanders is consistent with the feminizing influence of prenatal estrogen reported earlier by Yalom, Green, and Fisk (1973). In the present study there was no measurable effect of DESDP on the behavior of male offspring; however, sensitive tests for a possible feminizing influence were not incorporated in the present experimental design. The failure to find any “feminizing” or “demasculinizing” effects in males is at least consistent with a masculinizing role for the hormone, and such a role would certainly be predicted from the work with many nonprimate mammals. However, effects of estrogen observed in nonprimates do not always apply to human beings. To the obvious example cited earlier regarding effects on ovarian function, we should add the strikingly different results of androgen insensitivity in rodent and human males. Androgen insensitivity (AI) is a genetic abnormality in which androgen receptors are not synthesized, but estrogen receptors are. This means that the aromatized products of androgen metabolism are free to act, and they do so in an environment that is free of androgenic influence. The physical consequences of this genetic defect in human beings on the anatomical level are a female habitus at birth and throughout life, the development of breasts at puberty, and the absence of pubic and axillary hair. The psychological orientation is female in adulthood, and, so far as has been determined, also female during preadolescence and adolescence (Money, Ehrhardt, and Masica, 1967; Money, Schwartz, and Lewis, 1984). “Tomboyism,” characteristic of human females with adrenal virilizing syndrome, is not a characteristic of AI human males that look and act like females. In rats, anatomical findings parallel those for humans with small modifications for the signs of puberty, but the behavioral consequences are quite different. As one would expect in rodents, the afflicted animals show masculine behavioral traits reasonably well, and the feminine behavioral systems are suppressed. Thus, overall, the effects of prenatal estrogens derived from aromatization on psychosexual traits in rats and humans are nearly opposite.

ACKNOWLEDGMENTS

We gratefully acknowledge support from the National Institutes of Health (RR00167) to the Wisconsin Regional Primate Research Center and from NSF(U.S.)/CNRS (France) which provided fellowship funds to B.L.D. Our special thanks are due to Steve Eisele for his assiduousness in producing the necessary infants from the breeding colony, and to his wife, Carol Eisele, for her work in collecting the data and her dedication to the needs of the monkeys.

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