Effects of Cryptorchidy, Parabiosis, and Estrogen Administration upon Leydig Cell Tumorigenesis in Fischer Rats

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ABSTRACT

The effects on Leydig cell tumorigenesis of surgical cryptorchidy, parabiotic union with a castrate male partner, and a combination of the two procedures as well as of the continuous exposure to a high level of diethylstilbestrol stimulation were studied in the high-tumor Fischer rat. Localized foci of adenomatous hyperplasia were found to have developed in the scrotal testes of most animals by 13.5 months of age. Parabiosis with a castrate male partner did not appear to alter the process significantly, but surgical cryptorchidy, even when combined with parabiosis, completely prevented the development of this pretumorous lesion as did the chronic administration of diethylstilbestrol. Furthermore, intraabdominal residency throughout the life span of animals was found to inhibit Leydig cell tumor formation almost completely. Comparative end organ weights as well as plasma testosterone determinations reported earlier indicated that, with continued intraabdominal residency 3 months and longer, the Leydig cells in this strain of rat became significantly less responsive to stimulation by pituitary gonadotropic hormones.

The data are interpreted as indicating that the endocrine stimuli most centrally involved in the genesis of this tumor type are quite different in the mouse and the rat, an abnormal response to gonadotropic stimulation being pivotal in the rat while reaction to estrogens is the most significant factor in the mouse.

INTRODUCTION

Over the past several years, a considerable body of information has been amassed relating to neoplasms of the testicular Leydig cells in both mice and rats, and, frequently, attempts have been made to equate the findings in these murine species. Although the microscopic morphology and secretory activities of the neoplasms are quite similar in the 2 species, there are major differences in the circumstances under which they may be encountered. They occur spontaneously with significant frequency in older males of at least 3 strains of laboratory rat (10–12, 14, 21), while they are seen only rarely in the inbred strains of mice in common usage (3, 13, 18, 32). As will be covered rather thoroughly in "Discussion," this usually nonmetastasizing neoplasm has been produced in high frequency in a number of rat populations in various laboratories by several experimental manipulations. On the other hand, with the exception of small tumors produced by surgical cryptorchidy in BALB/c mice (20) and by administering cadmium to one noninbred strain (16) but not to another (34), the long-term administration of estrogically active compounds has been the only successful method of producing the often large metastasizing neoplasm in genetically susceptible mice (3, 13, 18, 19). These differences suggest that the underlying "causative" stimuli responsible for the neoplastic transformation of this cell type may, in fact, be quite different in these 2 closely allied species.

The purpose of this communiqué is to explore this possibility in some detail, presenting the results of a series of experiments that used Fischer rats in which the incidence of the spontaneous neoplasm is exceptionally high in aged animals (10, 12, 21). The experiments were undertaken initially in an attempt to shorten the latent period of the spontaneously occurring malignancy in an effort to gain insight into the influences that might be responsible for the development of the tumor. Since situations producing hypergonadotropism had been shown to induce Leydig cell tumors in rats of other genetic backgrounds, we tested the effect of relatively mild hypergonadotropism produced by parabiotic union to a castrate male partner. Surgical cryptorchidy, which in the BALB/c mouse has been shown to induce Leydig cell tumors in itself and to increase the tumorigenic effects of low-dose estrogen treatment (20), was tested alone and in conjunction with parabiosis. Finally, the effect of a high level of estrogenization with DES was investigated. When none of these procedures was found to shorten the latent period of tumor development significantly, the long-term effect of cryptorchidy was examined, and this was found to inhibit tumorigenesis most strikingly.

MATERIALS AND METHODS

All rats were from the inbred colony at the AMC Cancer Research Institute and Hospital that was started in June 1962 from 5 litters of pedigree Fischer (F344) animals graciously given us by Dr. W. F. Dunning, Cancer Research Laboratory, University of Miami, Coral Gables, Fla. Strict inbreeding via sibling matings was observed throughout the course of these experiments. The animals were housed in stainless steel cages on granulated corn cob bedding in an amply windowed dormitory, kept at 23.5–26.5°, and maintained on Purina laboratory chow and tap water ad libitum.

The first stage of surgical cryptorchidy was carried out when males were 4 weeks of age. This consisted of making a low-ventral abdominal skin incision and, by blunt dissection, separating the scrotal musculature from the overlying integument bilaterally. The gubernaculi were then fracted so that the

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The abbreviations used are: DES, diethylstilbestrol; SV, seminal vesicular-coagulating gland complex.

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testes could be delivered into the abdominal cavity. The cremasteric musculature and the processus vaginalis testis were excised at the abdominal wall, the cord structures were freed to the region just dorsal to the seminal vesicles, the muscular incision was closed with wound clips. Two weeks later, the abdomen was entered through a small midline incision in the epigastric area, the epididymal fat pads were located, and a small portion of each was delivered through the incision to be incorporated in the silk sutures used to close the abdominal musculature. This fixation of the epididymal fat pads to the ventral belly wall was found necessary in order to prevent subsequent torsion of the testicular pedicle resulting in vascular occlusion and testicular necrosis.

Parabioses were carried out during the seventh week of life essentially as outlined by the method of Bunster and Meyer (8) with the coelomic anastomosis being approximately 1 cm in length. Each parabiosed pair was housed with a single normal male acting as a "nurse" to assist in keeping the pair well groomed.

Estrogenization was accomplished through the s.c. implantation of 10-mg fused DES cholesterol pellets (1:9, w/w) prepared as described by Wieder and Shimkin (41). In order to approximate dosage used in our laboratory to produce Leydig cell tumors in susceptible strains of mice (18), one pellet for each 30 g of body weight was implanted at 8 weeks of age when the average weight was 182 ± 20 g.

All animals were inspected at least weekly and weighed every other week. Since the first macroscopically evident Leydig cell tumor had occurred in a 16.5-month-old male Fischer rat in our initial experiment (21), it was thought that, if either cryptorchidy, parabiosis, or the combination was to augment tumorigenesis significantly, the effect should be evident 12 months after the procedure was carried out, i.e., when the animals were 13.5 to 14 months of age. The largest number in each group, therefore, were allowed to live to this age, while smaller numbers of control and cryptorchid animals were sacrificed at 6 weeks, 3 months, and 6 months postsurgery in order to develop a notion of the continuum of changes that were occurring. In the case of the DES-treated animals, the experiment was terminated 10 months after pellet implantation at a time when pituitary enlargement was becoming quite pronounced.

At necropsy, weights of the testes, the SV, the ventral prostate, and the hypophysis were obtained. Testes, adrenals, and pituitary glands were fixed in Bouin's solution containing 1% acetic acid and stored in 80% ethanol until processed. When preparing the testes for histological examination, 4 to 6 blocks of tissue, each representing a complete transverse section, were made of each gland with multiple sections of each block cut at 5 μm for mounting on the slides. The tissues were routinely stained with hematoxylin and eosin except for those of cryptorchid testes where alternate slides were stained by a periodic acid-Schiff procedure in order to identify the altered tubular structures more clearly.

RESULTS

Body and Organ Weights. Cryptorchidy did not influence the weight gain of the animals during the first year so that at 13.5 months of age, the weight of the cryptorchid males averaged 343 g (n = 44), while that of the controls averaged 337 g. Although the parabiotic pairs appeared in good condition throughout the experiment, their gain in weight was subnormal. The mean weight at autopsy of the intact-castrate pairs (n = 41) was 506 g (253 g/animal) as compared to 538 g (269 g/animal) for the cryptorchid-castrate pairs (n = 40). In contrast, the 40 estrogenized males gained but little weight (only 30 g on the average) over the 10-month period of observation, having a mean weight of 203 g at autopsy.

The more significant organ weights are recorded in Table 1. With the exception of that of the ventral prostate in the 1-year nonparabiosed animals, the weight reduction of both the SV and ventral prostates in the cryptorchid groups more than 6 weeks postsurgically is statistically significant (p = 0.002 or less). It should be noted that, although these "androgen target" tissues of the animals with scrotal testes were larger in the parabionts, they were significantly smaller in the parabiosed cryptorchid animals than in the cryptorchid single ones. That the parabiotic unions were effective was evidenced by the fact that the SV weight of the castrate partners averaged only 22 mg, while their ventral prostates were too small to be dissected with any degree of accuracy. In general, these observations are in accord with the plasma testosterone levels as determined by Lloyd (31) on selected individuals from these populations. It is also of interest that, after 3 and 6 months of cryptorchidy, the pituitary glands had enlarged significantly (average, 11.4 mg in cryptorchids versus 9.6 mg in controls). After 1 year of parabiotic union, the hypothymseal weights of cryptorchid partners averaged 11.6 mg, and those of the scrotal animals averaged 8.2 mg, while those of the castrates averaged 15.3 mg.

Morphological Observations. No abnormalities were evident in the scrotal testes during the first 7.5 months of the experiment. After only 6 weeks of cryptorchidy, however, the germinal epithelium had been greatly reduced in thickness and consisted mainly of spermatogonia and primary spermatocytes in early stages of maturation supported by Sertoli cells (Fig. 2). The peritubular membranes had begun to thicken and become fissured. Because of the decreased diameter of the tubules, the interstitium was more prominent and frequently edematous, but the individual Leydig cells did not appear to have been altered significantly. Tubular atrophy continued, accompanied by thickening of the peritubular membranes and an increase of collagen in the interstitial spaces. By 6 months postsurgery...
cells were encountered in the numerous (over 400) sections characterized consistently different. A study of testes from dent. The Leydig cells were rather small, and their nuclei were thickened as in the cryptorchid animals, and the interstitial (3 of the unoperated controls and 3 of the parabionts). These often separated one from another by collagen. (Fig. 3), the Leydig cells had definitely decreased in size due whether or not these animals had been parabiosed.

After 12 months of cryptorchidy, the testes of the single and parabiosed animals also were indistinguishable one from the other. The tubular atrophy was extreme, and the peritubular membranes were thick and irregularly laminated (Fig. 4). The interstitial spaces were usually edematous, containing septa of collagenous tissue in which rather small Leydig cells were encased. No adenomatous nodules of hypertrophic Leydig cells were encountered in the numerous (over 400) sections studied.

The small testes from the 10-month-old DES-treated animals presented an entirely different microscopic picture (Fig. 8). Although the tubules were of small diameter, primary spermatocytes were abundant. The peritubular membranes had not thickened as in the cryptorchid animals, and the interstitial spaces were relatively small with no increase in collagen evident. The Leydig cells were rather small, and their nuclei were somewhat condensed. Again, adenomatous foci of hypertrophied Leydig cells were not encountered in any of these testes. Histological changes noted in the hypophyses are of some interest. As would be expected, the glands of the castrate parabiosed partners contained numerous large "castration" cells. However, a scattering of these cells was also readily identifiable in the hypophyses of the cryptorchid animals 3 months and more after surgery, a finding that might have been suspected from the significant increase in weight noted whether or not these animals had been parabiosed.

**Long-Term Cryptorchidy Study.** The 36 animals used for this portion of the study were operated upon at the same age as those in the shorter-term portion but were allowed to survive until they were obviously ill health. There were 2 early deaths (before 500 days); all but one (age, 620 days) of the remaining animals were necropsied, and 31 were sacrificed when considered moribund. As diagrammed in Chart 1, their median survival was 776 days (mean = 752), which was some 2 months longer than had been noted for normal males in the previous experiment (21). According to the rate at which macroscopically Leydig cell tumors had developed in those males, 27 of these cryptorchid animals should have developed the neoplasm (18 of them bilaterally), however, only 2 rather small tumors were found. It is of interest that the largest and most pleomorphic of these occurred in one of only 3 testes that had become strangulated due to torsion of the vascular pedicle.

The morphology of the nontumorous testes was extremely abnormal (Fig. 6). The peritubular membranes had thickened greatly with a layer of collagen interposed between the periodic acid-Schiff positive lamina and the basilar portion of the Sertoli cells. The interstitialia were composed mainly of vessels and collagenous tissue with small Leydig cells generally lining fluid-filled clefts, although a few remained embedded between the collagen fibers. One to 5 foci of Leydig cells, a few measuring up to 1 mm in diameter, were found in one-half of the testes. The individual cells making up these nodules were of small size (Fig. 7), much as the Leydig cells in the remaining testes, and did not appear hypertrophied as did those typically constituting the adenomatous nodules in the scrotal testes of younger animals.

**DISCUSSION**

It seems reasonable to conclude from these data that the endocrinological stimuli for Leydig cell tumorigenesis are, indeed, quite different in the two murine species. Surgical cryptorchidy not only failed to augment tumor formation as it does in BALB/c mice (20) but actually was strongly inhibitive of the spontaneous development of the neoplasm in Fischer rats. Similarly, the administration of a large dose of DES not only failed to result in the early development of Leydig cell neoplasms but also did not produce the diffuse Leydig cell hyperplasia seen early in tumor-susceptible strains of mice and appeared to inhibit the development of the pretumorous adenomatous foci of Leydig cells that are seen normally in this strain of rat. Although the period of estrogen therapy was shortened to only 10 months because of the development of significant pituitary enlargement, this should have been an ample period of exposure since Reddy et al. (33) reported that, in their laboratory, 80% of Fischer rats had developed Leydig cell tumors 1 year after the administration of cadmium.

The abnormal situations that have been reported to result in
the development of a high incidence of Leydig cell tumors in various rat populations are summarized in Table 2. Prolonged hypergonadotropism appears as a significant and unifying feature in the majority. In the experiments reported in this communication, parabiotic union with a castrate male partner did not shorten the latent period of tumor development significantly, although this procedure has been reported recently as resulting in the development of the neoplasm in older animals of a breed that does not ordinarily develop the tumor (7). Surgical cryptorchidism, however, inhibited tumorigenesis very significantly in the development of the neoplasm in older animals of a breed or of frank tumors of Leydig cell origin. Consideration should be given to the role of these endocrine factors in the spontaneous development of this tumor type. In the only strain of mouse in which a high incidence of Leydig cell tumors has been reported as occurring spontaneously (4), the males frequently develop cancers of the mammary glands as well as strongly suggesting an elevated level of estrogenic stimulation. The data obtained in the present studies of surgical cryptorchidism suggest that, in the rat, the actual level of gonadotropic stimulation may not be the only factor important in Leydig cell tumorigenesis. In addition, the ability of that cell to respond to the tropic stimulation seems to play a very significant role. Thus, the level of circulating gonadotropins need not be inordinately high in those breeds of rats that develop frequent neoplasms spontaneously if the response of the Leydig cells to tropic stimulation is abnormal or excessive.

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ADDENDUM

Adrenal Medullary Tumors. Ample histological material was available for study of the adrenal glands bilaterally in 25 of the 27 long-term cryptorchid animals that lived beyond 2 years of age. Criteria, essentially as proposed by Gillman et al. (15), were used to distinguish areas of focal hyperplasia from pheochromocytoma, and the material was reviewed independently by Dr. R. L. Swamy, Laboratory of Experimental Pathology, Hoffmann-LaRoche, Inc., Nutley, N. J. We were in agreement that all animals had adrenal medullary pheochromocytomas and that in all but 2 this lesion was evident bilaterally. The medullary tissue in 2 glands was very abnormal but was believed to represent extensive multifocal nodular hyperplasia rather than pheochromocytoma. In one animal, a 5-mm mass was found lying on the inferior vena cava at the junction of the left renal vein. This was composed entirely of pheochromocytoma tissue, but it was uncertain whether it represented a metastasis or the independent development of a lesion in an organ of Zuckerkandl.

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