The Incidence of Adrenal Cortical Carcinoma in Gonadectomized Female Mice of the Extreme Dilution Strain

I. Observations on the Adrenal Cortex*

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For some time now studies have been in progress in this laboratory on the relation of gonadectomy to changes in the adrenal cortex and to the development of the secondary sex organs that occurs following certain changes in the cortex.

In the dilute brown strain of mice (JAX dba), nodular hyperplasia of the adrenal cortex following gonadectomy has been observed and described (12, 31, 33). This change occurred in castrated male and female mice both, and preceded and then paralleled an increased development of the secondary sex organs. This development was in a female direction in both sexes, and mammary carcinomas were observed in both males and females that had been gonadectomized at birth. No gonadal regeneration was observed.

It was then shown that these adrenal and sexual changes were not uniform from strain to strain (30). Strains normally with a high, and others normally with a low incidence of spontaneous mammary carcinoma, were observed in this regard. Two strains, dba and C3H, with a high incidence of mammary carcinoma, have more extensive adrenal cortical hyperplasia and accessory sex organ development than does the C57 black strain, which has a low incidence.

The study of adrenal changes and their relationship to accessory sex organs was then extended to include a number of other inbred strains. It was a fortunate circumstance that a number of these had already been developed or were being developed at this laboratory. This report is based upon the extreme dilution strain (JAX cc), in which the adrenal and subsequent sexual responses are characteristically different from those of animals of the dilute brown strain, or of any other inbred strain studied and reported on thus far. It is increasingly evident that the response to gonadectomy varies greatly from strain to strain.

Removal of the testes or ovaries from individuals of the extreme dilution strain of mice leads to carcinoma of the adrenal cortex (32). As will be shown later, these carcinomas metastasize, are transplantable, and then cause increased development of the secondary sex organs of the host. Spontaneous carcinomas of the adrenal cortex are rare in mice, and except for a few cases of carcinomatous changes in our early gonadectomized dilute brown mice accompanying nodular hyperplasia of the adrenal cortex, they have not been observed in the Jackson Laboratory's colonies.

Review of the Literature

Slye (19) reported finding 4 adrenal tumors in 33,000 autopsies on mice. These were presumably spontaneous in uncastrated mice, as no mention is made of castration or any other treatment. One was in a male, and 3 were in female mice. Of these 3, the first was a "Cortical adenoma of misplaced interrenal adrenal rest." It was "composed of a solid mass of cells resembling those of the adrenal cortex except in lack of orderly arrangement, closely packed together, and flattening out a thin shell of adrenal cortex." No medullary elements were found. The second tumor was a "Mesothelioma of adrenal with peritoneal metastasis." Microscopically this showed "the usual features of the typical mesotheliomas . . . with slight

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1 The relative percentage incidence of these in males and females is not given.
tendency to alveolar arrangement.” The right adrenal was surrounded but not invaded by the tumor, and seemed normal. The retroperitoneal nodes were replaced entirely by tumor tissue. The third growth was a “bilateral malignant mesothelioma.” Each adrenal was about the size of the kidney. “... the gross appearance suggested general lymphosarcoma... seemed normal. The retroperitoneal nodes were replaced entirely by tumor tissue. The third growth was surrounded but not invaded by the tumor, and had a “bilateral malignant mesothelioma.” The right adrenal was about the size of the kidney. “... the neoplastic tissue was composed of a solid growth of cells with considerable cytoplasm and nuclei that varied greatly in size. Mitotic figures were abundant. The invaded lymph nodes were largely replaced by tumor cells, and tumor cells were found in the lungs also. The ages of the mice and the strain in which these growths arose were not given. Selye found no records of adrenal cortical neoplasms in mice in the literature.

Gardner (13) reported “adrenal tumors” in 13 mice ovariectomized when 43 to 65 days old, and 495 to 726 days of age at death. These mice were from the third and fourth inbred generations of the NH strain, a strain originating from the combination of 3 inbred strains and “developed for the purpose of producing the highest possible degree of biological variability without the interfering incidence of spontaneous tumor” (20). Some similarity of ancestry, although remote, may exist between the NH and ce strains. Evidence is being gathered on this point. These adrenal tumors were associated with evidences of estrogenic stimulation, as determined by the uterus, pelvis, and mammary glands. The size of the growth could be roughly correlated with evidences of high or low levels of estrogenic stimulation.

Dalton, Edwards, and Andervont (4) have reported a spontaneous, transplantable, adrenal cortical tumor in a female strain C mouse 24½ months of age. They presented evidence that this neoplasm originated from relatively undifferentiated cells of the adrenal cortex. The failure to find metastases from either the original growth or the transplanted tumors suggested that the neoplasm was relatively benign in character. However, the rapid growth rate attained in later generations plus invasion of the capsule indicated a certain degree of malignancy. Careful examination for gross and histologic changes, particularly in the urogenital system and adrenal glands, were made. In female hosts focal edema was noted in the deeper layers of the endometrial stroma; no changes were noted in the genital system of male hosts.

MATERIAL

This report is based upon females of the “extreme dilution” strain of mice, which originated in this laboratory and has now been inbred for from 25 to 30 generations. A preliminary report of the origin, breeding behavior, and tumor incidence in this strain was given at the Third International Cancer Congress, Atlantic City, 1939 (29). Since the proceedings of this congress were not published, some of the findings will be reviewed here.

As now constituted the strain is black agouti, homozygous for the albino series allele c e, a gene which, when homozygous, causes yellow pigment to be completely suppressed and black diluted to a light tan. The eyes remain dark. The coat of the young is much lighter than that of the adult. The identifying c e gene in this stock traces through very few hands to a mutant wild male trapped by Mr. J. E. Knight of Weldon, Illinois, in 1920. Detlefsen (7, 8) reported on the genetic behavior of this gene, and our attention was first drawn to some inbred animals carrying it because of evidence indicating that they might develop into a low mammary tumor strain. This has proved to be true. No mammary tumors have been found in several hundred breeding females maintained to advanced ages. In addition we secured evidence that there was a relatively high incidence of gonadal tumors in this strain, ovarian tumors constituting 33 per cent of the total tumors of the female.2 These mice also failed to reproduce efficiently; over 50 per cent of the females failed to breed when mated brother-sister.

Eaton (9) measured fertility, viability, and growth in 9 inbred strains of mice. One of these (c e 726) involved mice selected from strain ce after 15 generations of inbreeding. He found this ce substrain low in its percentage of successful matings, but highest of the 9 in number of young born per litter. The number of young raised and the weight of these at 120 days compared favorably with the other strains tested. A histochemical difference between “high” and “low” mammary tumor strains was found by Vicari (24). In this study of the amount and distribution of visible cortical lipids in the adrenal glands in relation to mammary tumor incidence she found the ce strain at one month intermediate between the dba strain and C57 blacks both in actual width of the sudanophile zone and width of the sudanophile zone in relation to the whole cortex. Following the work of Miller and Riddle (18) she presented the theory that the visible lipids are storage lipids. Thus ce mice would have less demand for adrenal cortical lipids than those of strain dba, and more than the C57 blacks, if the implications of her interpretation are applied. A peculiar finding of Vicari was that although in strains C57 black, dba, A, N, and C3H the

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2 Data to be reported in detail in a later article.

3 This failure to reproduce may well be due to the male or the female or to both. With some brother-sister mating groups only a few females produced young, while in other of these groups either all or no females had offspring.
Woolley and Little—Adrenal Cortical Carcinoma in Spayed Females. 1

male had actually as well as relatively more lipid material than the female in the adrenal cortex at 1 month, males and females of the cc strain were the same on a percentage basis, and actually the females had slightly more lipid than the males. The X zone was lipid-free except for a few scattered groups of 2 or 3 large lipid droplets in the females.

This report concerns only animals up to 13 months, that is, 26 virgin females and 34 ovariectomized mice. The number of animals at each monthly interval is given in Tables I and II. The findings for a group of castrated and control males are presented in a companion report (34).

METHODS

The technic used in this experiment was simple: A group of virgin controls was compared with a group of females ovariectomized when from 1 to 3 days of age.

The mice were anesthetized by chilling in the freezing compartment of an electric refrigerator for from 7 to 10 minutes; placing the mice on dry paper toweling aided in even chilling. For the operation they were placed on a cold glass plate. Small dorsal incisions were made over the region of each ovary with scissors and the ovary and capsule removed with the aid of small forceps; a single silk thread suture was sufficient to close the wounds. The operations were done with the aid of a dissecting microscope (7X ocular and 10X objective). Few mice were lost either as a result of the operation or of removal from their mother for the necessary period.

At 3 to 4 weeks of age the young mice were weaned, earmarked for identification, and maintained in groups of 4 on a diet of Purina fox chow and water.

### Table I: Observations on Strain CE Virgin Female Mice

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<th>No. of adrenals with carcinoma</th>
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### Table II: Observations on Ovariectomized Strain CE Mice

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* Microscopic to 0.5 cm. in diameter = small.
0.5 cm. to 2.5 cm. in diameter = medium.
Greater than 2.5 cm. in diameter = large.
Experimental and control mice up to 13 months of age were selected for autopsy at 30 day intervals and killed by cervical dislocation. Beyond 1 year of age the experimental mice were killed, either because of their large tumors or because they had attained a certain desired age. On account of this method of selection, as well as for ease of analysis, the data are divided according to age.

The adrenals were fixed in a mixture of alcohol, formalin, and acetic acid (11, page 89), embedded in paraffin, sectioned at 8 μ, and stained with hematoxylin and eosin.

Gross and microscopic observations were made on both adrenal glands of each mouse. When these were not enlarged sufficiently to give definite indication of extended neoplastic growth, they were serially sectioned.

Attempts to transplant a number of adrenal carcinomas were made, and in many cases were successful. This is to be the subject of a later report.

At autopsy a careful search was made to ascertain whether regeneration of the ovaries had taken place, and suspicious areas were sectioned.

**Observations on the Adrenal Gland**

**General.**—The normal adrenal gland of the mouse has been often described (11, 16, 25). In the young mouse at 30 days the cortex is divided into the following regions, from capsule to the medulla: zona glomerulosa; zona fasciculata, with a slight irregularity at the base of the columns; and the X zone, a large cosinophilic zone, relatively larger in the female than in the male, interlocking with the medulla. During the next few months the situation varies for males and females because of a difference in degeneration of the X zone, discussed in a later paragraph. In both males and females the X zone eventually disappears entirely, leaving only a connective tissue band between the cortex and the medulla, while the irregularity at the base of the columns of the fasciculata becomes known as the zona reticularis (25, 26). The adrenal cortex without the X zone has been termed the permanent cortex and the X zone the transitory cortex (27). When considering the question of growth, both normal and abnormal, it is of interest to recall that Whitehead (28) found mitotic figures commonest in the cortex immediately beneath the capsule of the gland and throughout the X zone during the first few weeks of the life of the mouse. Later in life mitotic figures were infrequent in all parts of the normal cortex.

**X Zone**

**A. Virgin females.**—At 1 month the X zone was intact; a few mitotic figures were present. At 2 months it was slightly hyperemic, with some evidence of cellular shrinkage near the medullary border. Mitotic figures were not seen in X zone cells then or at more advanced ages. At 3 months there was extensive nuclear and cellular shrinkage of X zone cells as well as extensive hyperemia in this zone. This proceeded irregularly from region to region within individual glands. The X zone was narrow for the next few months, and seemed completely gone in most regions at 7 months and all regions at 9 months.

At no time did the X zone occupy so extensive a part of the adrenal cortex as in some adrenals of virgin females from the strains used by Howard-Miller (16) or by Takewaki (22).

When the age at which the X zone degenerated was compared with the observations of Daughaday (5) it was noted that the adrenal glands of mice from the ce strain more closely paralleled those from the dba strain than those of C57 blacks. According to his observations, the X zone in C57 black mice cannot be identified as a distinctive region of the cortex after 100 days, whereas in dba mice it may persist to 210 days or longer.

**B. Ovariectomized females.**—The X zone at 1 month was similar to that in a control virgin female of the same age. Degeneration was evident at 2 months, and had progressed further than in controls of the same age. This acceleration of X zone degeneration was evident at later periods also, until at 6 months the process was complete. As degeneration proceeded there was more variation from region to region within individual glands than in the glands of control virgin females.

It has been pointed out (6, 27) that involution of the X zone may occur in two different ways; either it becomes vacuolated, the cells being loaded with fatty substances and degenerating in situ, leaving a mass of connective tissue stroma; or the cells merely become shrunken and crowded together and finally disappear. Waring (25) emphasized the fact that fatty degeneration is never found in the male. Involution in ce mice was almost entirely without vacuolation.
and fatty degeneration. Variation in the method of X zone degeneration exists between strains of mice, since Daughaday (5) found more vacuolation in the high mammary tumor dba strain than in the low tumor C57 black strain.

Beginning at 5 months in ovariectomized females, and at 6 months in control virgin females, groups of cells with yellow pigment and vacuoles, as well as giant cells containing yellow-brown pigment, vacuoles, and 2 to 14 or more scattered nuclei, were found near the cortico-medullary border. Similar giant cells have been described under the term "brown degeneration" (3). These became more numerous in older age groups, but never formed a continuous band or ring. They first appeared when X zone degeneration was nearing completion. It has been reported that the development of these cells tends to be greatest in mice of advanced age, but the time of earliest occurrence was not determined (1). It has been suggested that these cells have some relation to the etiology of mammary gland carcinoma (2), but this has been questioned (1). The extent of development of brown degeneration varies from strain to strain (1, 2, 17). It has been produced by the prolonged application of estrogens (2).

"Type A" cells.—Attention is called in this paper to groups of subcapsular cells that usually have not been emphasized in discussions on the architecture of the mouse adrenal. Localized groups of these cells occurred in both intact and ovariectomized mice, where the cells could be identified on gross and microscopic examination by the time the animals were 2 months of age. The areas became more numerous and more extensive as age advanced.

In the gross they appeared as round, dark areas that seemed to be scattered at random over the adrenal; later, depending on other conditions within the animal, they occupied little or much of its surface. For convenience the cells composing these areas will be termed "type A" cells (Figs. 2 to 5).

Microscopically, foci of type A cells were first found in the immediate subcapsular region of the cortex. The cells were usually ovoid, although they varied from round to slightly fusiform. The nucleus was generally ovoid and relatively large. The cytoplasm stained very slightly and the cell walls were indistinct. These cells seemed to be similar in character but not in extent to the first subcapsular cells described by Zwemer, Wotten, and Norkus (35) for the mammalian adrenal.

Foci of these cells were perhaps the result of an increase in subcapsular cells without subsequent, or at least rapid, differentiation. This interpretation assumes continuity of the cell types from the capsule in the growth of the cortex from without inward.5

In older virgin females type A cells were found massed 6 to 8 layers deep under the capsule to the exclusion of other cells. They also surrounded groups of zona glomerulosa cells, and penetrated in groups 3 to 4 cell layers wide between the cell columns of the zona fasciculata. In general, expansion of these areas was inward toward the medulla as age increased (Fig. 4).

In ovariectomized females, foci of type A cells were more extensive at any given age than in virgin females, and also they were definitely localized. In an ovariectomized mouse 4 months of age the cells had massed some 15 layers deep to the exclusion of other types in localized areas. Beginning at 4 months of age foci of type A cells were themselves disturbed by enlarged cells, and at 6 months and later by adrenal cortical carcinomas. At 11 and 12 months only adrenal glands were without these neoplasms; 1 in 4 P2273 and 1 in 4 P2214.6 In both, masses of type A cells were in general limited to the outer third of the adrenal cortex, and were very numerous in this region. In 4 P2273 the adrenal capsule had been ruptured and type A cells had invaded the surrounding fat.

There was a relation between focal degeneration of the X zone and localized hyperplasia of the subcapsular cells. Type A cells appeared near the capsule over regions where X zone degeneration was rapid. In a few locations, at 2 months, X zone degeneration preceded the appearance of localized masses of type A cells, though at most sites both changes occurred at this age. The relationship was followed until eventually X zone degeneration was general. Both the localized degeneration and the grouping of type A cells were more pronounced in ovariectomized than in virgin females. The cytoplasm of the cortical cells between the type A cells and the degenerating X zone cells stained more deeply with eosin than similar cells in other regions of the same gland. This probably indicated a metabolic disturbance.

Attention has been called to type A cells not only because (a) they were more prominent in ovariectomized than in virgin mice and (b) more definitely localized in the former than in the latter, but also because (c) they were related to focal changes in the adrenal cortex, possibly to the location of the small cortical carcinomas to be described later.

"Type B" cells.—In the adrenal glands of ovariectomized mice, beginning at 4 months of age, certain

4 Distinguished from subcapsular cells of the zona glomerulosa described in the same article.

5 Further study of the method of cell replacement in the cortex of the mouse adrenal seems to be needed to establish the relative importance of different possible methods. Perhaps extensive differentiation of subcapsular cells into other types is not to be expected here.

6 Ovariectomized females.
cells were found greatly enlarged. Their cytoplasm took relatively little stain. Groups of these cells were localized within or close to the groups of type A cells, already described (Fig. 6).

Areas containing these cells could be observed upon gross examination as dense white spots on the surface of the adrenal, encircled at first by the darker looking type A cells. They increased in size as age increased. By 12 months the white color changed to yellowish-brown.

Microscopically, these enlarged cells (for convenience termed "type B" cells) were found localized within or adjacent to masses of type A cells. They were polygonal in shape, irregular in size and arrangement, their cytoplasm stained lightly with eosin, and vacuoles were present. The nuclei were slightly larger than those of the zona fasciculata. When type B cells were in the region normally occupied by zona fasciculata, they did not show the variation in density of staining often observed in the cytoplasm of its own cells. By 6 months all layers of the cortex were interrupted by groups of type A and type B cells, and in a few cases at 6 and 7 months the capsule had been raised by masses of these cells; i.e., the adrenals were nodular.

Areas of type A and type B cells were also noted in our series of gonadectomized dba mice. Extensive masses of these cells formed areas that were described as nodular hyperplasia. In the ce strain these areas, although less extensive than in dba mice, took on new significance because in many cases they were spatially related to and completely enclosed small carcinomatous areas. Fig. 8 shows a small carcinoma among enlarged cells.

Why should the ovariectomized mice have hypertrophy of the adrenal cortex? Applying the speculative method of Tepperman and his associates (23) to an interpretation of the present type of hypertrophy one might assume that (a) there had been, for some reason, an increased inactivation (utilization) of adrenal cortical hormone because of ovariectomy; (b) the fall in adrenal cortical hormone level removed an inhibitory influence on the adrenotropic activity of the anterior pituitary; (c) increased adrenotropic activity led to hypertrophy of the adrenal cortex. The intimate relationship between the pituitary and the adrenal cortex has been reviewed (21).

The antecedents of type B cells are not known; perhaps these originate from glomerulosa cells. At least they were often found in the glomerulosa zone. Another possibility is that they originate from type A cells that preceded them in time of appearance and later were in close spatial relation to them.

**Carcinomas of the Adrenal Cortex**

**A. Frequency.**—Tumors of the adrenal cortex first appeared in 100 per cent of the ovariectomized females at 6 months of age, and were present in 100 per cent of the females in succeeding age groups. In this period, 6 to 12 months inclusive, 21 ovariectomized females were examined (Table II). In the same age group 11 intact females were examined and no adrenal cortical tumors were found. Thus these tumors, a universal characteristic among the older ovariectomized mice, were limited entirely to this group. Among the 21 females with carcinomas the growths were bilateral in 11. Since a number of the growths were found only upon microscopic examination, and no record was kept of right and left adrenals where no tumor was obvious, the frequency of tumors for each side alone was not determined. The frequency of these growths as a whole shows an increase over that previously reported (32) because more small tumors are included.

**B. General appearance.**—In general the smaller carcinomas were dense, cellular nodules composed of atypical cells showing frequent mitotic figures. They were embedded in the outer region of the cortex, which already was disorganized with groups of type A and type B cells (Fig. 8).

Expansion into the medulla was evident in all cases as the nodules enlarged (Fig. 9). Eventually the medulla and the remaining noncarcinomatous cortical tissue was spread out more or less as a sheet a few
cells thick; first over much of the tumor and later, because of its size, over part of its outer surface (Fig. 10). The growth was fairly well rounded in its earlier stages, but as it attained a size of 1.5 cm. or more in diameter protuberances often developed. The rounded contour was also modified by the pressure of the adjacent kidney. Not only did the kidney modify the shape of the tumor, but the tumor modified the kidney. In some instances this organ was flattened on its anterior surface; in others, partially covered over by tumor, but no invasion of the kidney was noted despite this close spatial relationship. The approximate sizes of the neoplasms, taking right and left tumors together, in different mice at their autopsy age are given in Table II. It seems evident that a mouse may carry an adrenal growth for many months without its causing death, for none died of cortical carcinoma up to 13 months of age.8

A detailed account of the structure of these growths will appear in a future publication.

C. Metastases.—One ovariectomized female at 12 months of age, P2271, had a metastasis to the lung, but none were found elsewhere in this animal, nor were any discovered in other mice at ages from 6 to 13 months. Dalton, Edwards, and Andervont (4) found no metastases from adrenal tumor C-199, and Gardner (13) none in his series of adrenal tumors in NIH mice. Ewing (10), however, refers to "bulky metastases" from diffuse adrenal carcinoma in man; Glynn (14) reports for man: "They do not all produce metastases."

DISCUSSION

The removal of ovaries from ce strain female mice was definitely associated with the formation of adrenal cortical carcinoma. Unless this operation was performed these tumors were absent. When the ovaries were removed, 100 per cent of the females from 6 to 12 months of age inclusive (21 in number) developed these neoplasms. It seems to have been the history of cancer research that progress has been made, with

8 Translating this roughly into terms of the human adult this would mean that should a patient have a tumor of this type and rate of growth he might be expected to survive at least 15 or 20 years. It is known, however, that children with untreated adrenal tumors often live only a very few years, and Ewing (10) states that most adrenal carcinomas progress rapidly from the first symptom.

SUMMARY

Two groups of mice belonging to the ce strain, and from 1 to 13 months of age, were autopsied at monthly intervals. The groups consisted of: (a) intact virgin females, and (b) gonadectomized females. Ovariectomy was performed when the mice were from 1 to 3 days of age. Adrenal cortical carcinomas were found only in the ovariectomized mice. These occurred in 100 per cent of the 21 females, 6 to 12 months of age inclusive, and in none of the 26 intact mice of this strain. Progressive changes in the adrenal cor-
tex preceding the appearance of the carcinomas are described.

REFERENCES


The Incidence of Adrenal Cortical Carcinoma in Gonadectomized Female Mice of the Extreme Dilution Strain. I. Observations on the Adrenal Cortex

George W. Woolley and C. C. Little


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