Morphology, Natural History, and Enzyme Patterns in Mammary Tumors of the Rat Induced by 7,12-Dimethylbenz(a)anthracene1

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SUMMARY

The natural history, morphology, and hormone responsiveness of 218 7,12-dimethylbenz(a)anthracene-induced breast tumors in the rat were observed and classified. The cell distribution and biochemical activity of nonspecific phosphatases and β-glucuronidase were studied. One-third of all tumors grew progressively, one-third regressed spontaneously, and one-third remained stationary in size over a prolonged period. Three histologic patterns were distinguished which reflected the degree of differentiation, and a fourth category was characterized by secretion-like changes which appear to be a response to estrogen stimulation. Histologic type was related to growth patterns which regressed following ovariectomy and those which did not, and these patterns were studied. One-third of all tumors grew progressively, one-third regressed spontaneously, and one-third regressed spontaneously. Alkaline phosphatase was localized in myoepithelial elements of the tumors, and the distribution of the enzyme was not altered after ovariectomy or estrogen administration. The activity of alkaline phosphatase was significantly reduced in static as compared to actively growing tumors. β-Glucuronidase was increased in a small group of tumors showing secretion-like activity histologically.

INTRODUCTION

The tumors induced in the rat breast by the administration of 7,12-dimethylbenzanthracene (DMBA), although generally hormone-responsive (10, 17), vary in their natural history and in their response to changes in hormone environment, and it would be of interest to know to what extent these variations are reflected in morphologic and biochemical patterns. In previous studies of these relationships there has not been agreement as to the extent of correlation between histologic features and growth patterns (15, 18, 19).

Biochemical investigations in carcinogen-induced breast tumors have been chiefly concerned with pyridine nucleotide-linked dehydrogenases (6, 12). In the present study interest is centered on several hydrolytic enzymes which probably play an important role in the metabolism of breast tumors. Alkaline phosphatase is present in high concentration in myoepithelial cells, which are a prominent component of both normal breast tissue and experimental breast tumors, and acid phosphatase has been reported as being abundant in human breast carcinoma (4). β-Glucuronidase is present in high concentration in target organs of estrogen stimulation, including uterus, breast, prostate, and adrenal cortex (5).

The purpose of the present paper is to define and describe the histologic types of the DMBA-induced breast tumor and to correlate these with the several growth patterns; to describe the effects of ovariectomy and estrogen administration on morphology and growth patterns; and to report the histochemical and biochemical distribution of nonspecific phosphatases and β-glucuronidase.

MATERIALS AND METHODS

Female Sprague-Dawley rats obtained from a commercial supplier were used throughout. These were obtained at the age of six weeks and were housed four to six animals to a cage and allowed Purina Lab chow and water at will throughout the experiment. Tumors were produced by administration through an intragastric tube of 20 mg of DMBA (Sigma Chemical Co.) dissolved in 1 ml of sesame oil, either in a single dose or in divided doses of 10 mg each with a 2-week interval between doses. In all animals the drug (or the first dose) was given at age 50 days. No significant differences in morphologic or biochemical features were noted between tumors from animals given DMBA in a single dose or in divided doses, and in the results described no distinction is made between these groups.

Tumors developed 4–6 weeks after the feeding of DMBA, and continued to appear at a decreasing rate during the experimental period. Twice weekly the animals were examined, the skin over the tumors was shaved, and each tumor was measured in three dimensions. A growth curve was drawn for each tumor relating duration to the geometric mean of the three measured dimensions. Tumors were removed under ether anesthesia at varying intervals so as to obtain tumors of various sizes and ages at different stages of growth. Samples from the most solid and apparently viable part of each tumor were fixed in 10% neutral buffered formalin and in Carnoy's fixative. Autopsies were performed on all animals except where this was precluded by postmortem change or

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mutilation by cage mates, and samples of major organs were taken for routine histologic examination. Histologic sections of tumors were stained with hematoxylin and eosin, toluidine blue, mucicarmine, Best’s carmine, periodic acid-Schiff with and without diastase digestion, and oil red-O. Bilateral ovariectomy was performed through separate flank incisions under other anesthesia. Estradiol-17β (Mann Research Laboratories) was given in daily subcutaneous injections in a dose of either 10 μg or 50 μg of the dry powder dissolved in 0.2 ml of a 10% alcoholic solution in sesame oil.

Histochemical preparations were made on fresh-frozen cryostat sections postfixed in 10% neutral buffered formalin at 4°C for 30 seconds. For biochemical determinations representative material from each tumor was minced and suspended in 0.12% aqueous solution of Triton X-100 (Rohm & Haas Co.) to make a final suspension of 100 mg tumor/ml. The suspension was homogenized in a Potter-Elvehjelm type apparatus and centrifuged at 25,000 × g for 30 minutes at 0°C. The precipitate was discarded and the supernatant assayed for β-glucuronidase activity by Kerr and Levvy’s (11) modification of the method of Talalay et al. (16), using phenolphthalein glucuronide (Sigma Chemical Co.) as the substrate. Nonspecific alkaline and acid phosphatases were determined by the methods described by Bergmeyer (1), using p-nitrophenyl phosphate as the substrate.

Data from biochemical analysis of enzymes were compared for the various histologic types and growth groups by analysis of variance; significant differences were determined by Scheffé’s procedure, using 95% confidence limits (2).

RESULTS

Morphology. During the period of observation 61 rats developed 218 breast tumors and these constituted the experimental group. Tumors occurred with equal frequency on the right and left sides of the body, but were found in the thoraco-cervical region more frequently than in the abdominointernal region in a ratio of 2.7/1. Tumors were always well circumscribed but not encapsulated, and infiltration into the adjacent muscle and fat was common, though never for a distance of more than 1–2 mm. Ulceration of the overlying skin frequently occurred in large tumors (30–40 mm diameter), but extension through the abdominal or thoracic wall was not seen, nor were any regional or distant metastases found. Many of the tumors contained small and large cysts filled with old blood or tissue fluid.

Of the 154 tumors examined histologically all were epithelial tumors; they appeared to be of ductal origin with varying amounts of intervening fibrous stroma and lymphocytic infiltration. Mast cells were prominent in the stroma of all tumors. Papillary structures lined by several layers of cuboidal epithelial cells were the predominant feature in many tumors, while in others these were inconspicuous or absent. Duct-like structures could be recognized as the basic component of the solid areas, and were lined by columnar or cuboidal cells and usually filled with fluid which was stained heavily positive by the periodic acid-Schiff method, both before and after diastase digestion, and lightly positive with mucicarmine. Glycogen could not be demonstrated in any tumor with Best’s carmine.

Within the general structural pattern described there were several variants which form the basis of a classification into four histologic types. The distribution of these is summarized in Table 1. The distinction between histologic types follows closely the classification described by Stevens et al. (15), and is based on the amount of epithelial cell proliferation around duct structures; this seems to reflect differences in degree of differentiation. Type A is a poorly differentiated tumor in which the few recognizable duct structures are surrounded by broad, irregular areas of epithelial proliferation. The tumor cells have cytologic features of immature cells, mitotic figures are numerous, and the histologic appearance is that of a highly malignant tumor (Fig. 1). In type B tumors, ducts are surrounded by only a few layers of epithelial cells and a pattern of ducts and small cystic spaces predominates. The peripheral layer of cells around the tumor nests is often distinct, and probably represents myoepithelial elements. Cells are of moderate size and mitoses may be many or few (Fig. 2). Occasional tumors of types A and B contain broad areas of sarcoma-like transformation, the epithelial cells breaking away from periductal tumor nests and growing as elongated, spindle-shaped cells without cohesion in loose, collagenous stroma (Fig. 3). In type C tumors the histologic appearance suggests atrophy, or at least a low level of proliferative and secretory activity. Duets are dilated and lined by only a single layer of small cuboidal or flattened cells. Fibrous stroma is abundant. Mitosis is usually absent (Fig. 4). Type D tumors are characterized by the presence of innumerable large and small intracytoplasmic vacuoles containing material which stains positively with oil red-O, negatively with periodic acid-Schiff, mucicarmine and Best’s carmine, and stains brownish-yellow in osmic acid-fixed, Epon-embedded sections. This feature was only observed in tumors showing evidence of active epithelial proliferation. In some type D tumors the glandular structure was well preserved, and the material from within vacuoles appeared to be extruded into gland lumens (Fig. 5). In other tumors the lipid-filled cells were crowded together and took on a trabecular arrangement, almost obliterating the ductal architecture and intervening stroma, and resembling secreting breast tissue in the late stages of pregnancy (Fig. 6). Besides the tumors designated as type D, occasional tumors of types A and B contained limited focal areas of similar lipid accumulation.

An important feature of many of the tumors was the occurrence of focal areas and nodules which differed sharply from the rest of the tumor in histologic pattern, so that lesions of
one histologic type contained nodules of a different type. It was usually possible to classify tumors on the basis of the predominant pattern, but some overlap between types was commonly seen.

Growth Pattern. Three growth patterns could be distinguished after the initial period of growth to a mean diameter of about 1 cm: those which continued to grow, those which reached a plateau and remained at about the same size for a prolonged period, and those which regressed. Those tumors which continued to grow or which regressed did so at different rates, and a few which showed a trend in one direction underwent more or less abrupt change to the other. Most tumors which regressed did so early in the period of observation or after a prolonged static period. Only a few regressed after prolonged growth, and spontaneous regression was not observed in any tumor which had reached a mean diameter of 2 cm (Charts 1, 2). In all of the animals studied there appeared to be a random distribution of patterns of tumor growth. In some rats with multiple tumors several or all of these were of the same pattern, while in other rats there was a random mixture of patterns.

Because of these variations a minimum observation period of 30 days was selected as necessary to establish the definitive growth curve of a tumor, except that in tumors which regressed to a diameter of 3 mm, or those which grew to 2 cm or over, a 3-week period of observation was deemed sufficient. Growth curves of 87 tumors fulfilled the above criteria and were considered to be "definitive" for the purpose of the study. Twenty-seven of these (31%) had a pattern of continuous growth, 29 (33%) remained in a prolonged static phase after initial growth, and 31 (36%) regressed. Some of the latter group disappeared and could not be used in morphologic or biochemical studies.

In 109 tumors both histologic features and growth pattern were studied, and histologic classification was assigned without knowing the growth pattern. Some of this group had been excised too soon to be included among those with a "definitive" growth curve, but in all instances a trend had been clearly established for at least 10 days before excision. The number of regressing tumors in this group is small because many of the tumors so classified had regressed to a small, 1-2 mm, fibrotic nodule at the end of a definitive observation period and were unsuitable for biochemical studies. Therefore, only 11 were sampled. Tumors reduced to this size were invariably of histologic type C. Histologic examination of the 109 tumors revealed only an approximate correlation with growth pattern, similar to that observed by Stevens et al. (15). About half of the tumors excised during an active growing phase were classified as histologic type A, whereas none of the regressing tumors fell into this category; only five type C tumors were found, of which three were in a regressing phase and two were stationary. Type B tumors formed the majority of the series (65% of the total) and formed a substantial number of the tumors in all growth categories. The data are summarized in Table 2.

Effect of Ovariectomy. Twenty-one additional rats bearing 71 tumors were subjected to ovariectomy and then divided into three groups, the first group receiving no further treatment while the second and third groups received daily injections of estradiol-17β, 10 μg and 50 μg respectively, beginning on the day of operation. Half of the tumors in each group were removed 2 weeks after surgery and the remainder after 4 weeks.

The effects of these procedures on the growth patterns and histologic features of the tumors are summarized in Tables 3 and 4. None of the tumors continued to grow after ovariectomy alone, and most regressed, while in the estrogen-treated group of 48 tumors 20 (42%) continued to grow, compared to 31% in intact, untreated, tumor-bearing animals.

The number of tumors is too small to allow statistically significant differences to be demonstrated in the various histologic subgroups, except for the occurrence of eight type D tumors among 39 tumors in the estrogen-treated group, compared to only four among 155 tumors in the intact animals previously studied. This suggests that the secretion-like change is a response to estrogen stimulation, while the occurrence of a similar histologic type in a few untreated animals may be due to an unusual response to endogenous estrogen.

Histochemistry and Biochemistry. Alkaline phosphatase was demonstrated histochemically in abundance in all tumors, and was localized in glandular lumens and in the peripheral one or two rows of epithelial cells in all of the periductal nests of tumor (Fig. 7). Occasional solid areas of tumor cells and areas of obvious cell degeneration also reacted positively.

Rat Mammary Tumors

Charts 1 and 2 show the static and diverse growth patterns of four tumors in a single rat.
Acid phosphatase was distributed sparsely and evenly throughout all cells of the tumors with no detectable pattern of localization, except for appearing in heavier concentration in macrophages and in areas of necrosis, and often in the cell debris within gland lumens.

β-Glucuronidase was frequently present in high concentration in histiocytes and fibroblasts of the stroma, but was often concentrated in many neoplastic cells within focal areas of tumor (Fig. 8).

The distribution and apparent concentration of the enzymes as shown histochemically did not vary significantly among the various histologic types and growth patterns, and did not seem to be affected by ovariectomy or subsequent estradiol administration.

Quantitative measurements of phosphatases and β-glucuronidase in the tumors are reported only for the group of intact rats. There was clustering of results around a median figure for each enzyme but with considerable variation between some individual tumors from low to quite high values. When the tumors were grouped according to growth pattern and histologic type, the concentration of alkaline phosphatase was significantly reduced in static as compared to actively growing tumors, and β-glucuronidase was increased in the small group of tumors showing secretion-like changes histologically (Table 5). Activity of the enzymes was not related to the size (wet weight) of the tumors, or to their age, measuring from the time of first appearance.

**DISCUSSION**

While confirming the work of previous authors that the DMBA-induced breast tumor in the rat is not a homogeneous population with regard to its natural history, the data in this study support the findings of Stevens et al. (15) that there is some degree of correlation between histologic features and growth pattern. Well-differentiated histologic patterns with atrophic epithelial changes were found only in static or regressing tumors, while regressing tumors contained no examples of poorly differentiated (type A) histologic patterns. The correlation disappears, however, in the large middle group of partially differentiated tumors, which was the predominant type in all patterns of growth. The presence of microscopic nodules which differ in histologic type from that of the surrounding tumor further precludes the use of cell pattern in predicting the natural history of individual tumors. Previous investigators (9, 19) noted the occasional areas of atrophic epithelium in otherwise cellular tumors, and suggested that cell groups within a given tumor probably vary in their responsiveness to the

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### Table 2

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Total</th>
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<tr>
<td>Growing</td>
<td>22</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>Static</td>
<td>9</td>
<td>40</td>
<td>2</td>
<td>1</td>
<td>52</td>
</tr>
<tr>
<td>Regressing</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>71</td>
<td>5</td>
<td>2</td>
<td>109</td>
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</table>

Growth pattern and histologic type in 109 7,12-dimethylbenz(a)anthracene-induced breast tumors in the rat.

### Table 3

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>Growing</th>
<th>Static</th>
<th>Regressing</th>
<th>Total</th>
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<tr>
<td>Ovariectomy only</td>
<td>0</td>
<td>3</td>
<td>18</td>
<td>21</td>
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<tr>
<td>Ovariectomy + 10 μg/day estradiol-17β</td>
<td>8</td>
<td>11</td>
<td>8</td>
<td>27</td>
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<tr>
<td>Ovariectomy + 50 μg/day estradiol-17β</td>
<td>12</td>
<td>6</td>
<td>3</td>
<td>21</td>
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</table>

Growth patterns of 69 7,12-dimethylbenz(a)anthracene-induced breast tumors in the female rat after ovariectomy and daily estradiol administration.

### Table 4

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovariectomy only</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Ovariectomy + 10 μg/day estradiol-17β</td>
<td>2</td>
<td>17</td>
<td>3</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>Ovariectomy + 50 μg/day estradiol-17β</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>15</td>
</tr>
</tbody>
</table>

Histologic types of 50 7,12-dimethylbenz(a)anthracene-induced breast tumors in the female rat after ovariectomy and daily estradiol administration.

### Table 5

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Histologic type</th>
<th>Growing</th>
<th>Graduating</th>
</tr>
</thead>
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<tr>
<td>Alkaline phosphatase a</td>
<td>23.7 ± 7.1</td>
<td>25.3 ± 4.2</td>
<td>28.7 ± 24</td>
</tr>
<tr>
<td>Acid phosphatase a</td>
<td>4.21 ± 0.44</td>
<td>3.83 ± 0.18</td>
<td>3.23</td>
</tr>
<tr>
<td>β-Glucuronidase b</td>
<td>117 ± 10</td>
<td>153 ± 8.0</td>
<td>143 ± 64</td>
</tr>
</tbody>
</table>

Enzyme activity in 7,12-dimethylbenz(a)anthracene-induced rat breast tumors in different histologic types and growth patterns. Figures represent means ± standard errors. Figures in parentheses are the number of tumors assayed.

a Expressed as μmoles p-nitrophenylphosphate/min/gm of wet tissue.
b Expressed as μg phenolphthalein/min/gm of wet tissue.

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hormonal environment. The observation of Rigden et al. (14) on the course of development of 3-methylcholanthrene-induced breast tumors in rats indicate that tumors form by coalition of clones of neoplastic cells arising from small ducts. It is likely that a similar course is followed in the development of DMBA tumors, and this would help to explain the nodular variants in cell type in some tumors, and the observed variation in hormone responsiveness. The atrophic epithelial changes observed in many spontaneously regressing tumors did not appear to differ from similar changes seen in tumors following ovariectomy, and in this respect our observations support those of Stevens et al. (15).

The secretion-like change in tumors from four of the intact rats and eight of the estrogen-treated rats is the most interesting and possibly the most significant histologic observation in these studies, since it represents, as Daniel and Pritchard have pointed out in a recent study (3), the only response to hormone stimulation which has so far been identified histologically in these tumors. These authors referred to the vacuolar change as milk-secretion, and noted a predominance of this histologic type in late-appearing DMBA tumors, i.e., tumors appearing about 40 weeks after administration of the carcinogen. The small proportion of secreting tumors in our material may be due to the shorter observation period, which extended over a total period of 20 weeks. Rees et al. (12, 13) noted the secretory change in both 3-methylcholanthrene and DMBA-induced breast tumors after administration of estradiol-17β to ovariectomized rats, and demonstrated an increased proportion of C-10 to C-14 triglycerides in extracts from secreting as compared to nonsecreting tumors (13), a biochemical feature which is also characteristic of breast milk. Young, et al. (19) mentioned the occurrence of "fatty change" in some of the tumors which they observed, and although these are not illustrated, the change may be the same. Hilf and his coworkers (7, 8) have described a secretion-like change of identical appearance in a transplantable mammary carcinoma in the rat following administration of estrogen. The change was accompanied by the appearance of casein and whey proteins in fluid extracted from the tumors, and by patterns of enzyme activity similar to those found in lactating breast tissue. The increased level of β-glucuronidase found in four tumors of this type in our own material is compatible with interpretation of the secretion-like phenomenon as an estrogen-related metabolic response, but more data are needed for confirmation. If specific milk proteins can be confirmed in the secreting type of DMBA tumor this will be, together with Hilf's work, a unique instance of hormone-induced differentiation in a neoplasm.

No answer is suggested by these observations to the question of why spontaneous regression occurs in such a large proportion of DMBA-induced breast tumors. Young and Cowan (18) failed to prevent regression of tumors by administration of estradiol-17β and progesterone. In our experiments, daily injection of estrogen at two different dose levels in castrated animals still resulted in regression of over 20% of the tumors. The observations support Young and Cowan's view that the regression is probably not due to higher estrogen requirement, although a different hormone combination or dose level might alter the result. It seems likely that differences within the tumors themselves rather than host factors are decisive in causing regression, since both regressing and rapidly growing tumors are often observed in the same animal.

The decrease in alkaline phosphatase activity in static tumors, as compared to those in active growth phase, appears to be statistically significant and may reflect a decrease in metabolic activity, but overlapping of a number of values found in individual tumors from the two groups indicates that the difference is of doubtful practical significance.

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Fig. 1. DMBA-induced breast tumor in the rat: histologic type A, poorly differentiated. H & E, × 530.
Fig. 2. Histologic type B, well-differentiated. H & E, × 425.
Fig. 3. Sarcoma-like change in a type B tumor. The tumor cells are epithelial, and many transitional areas can be demonstrated. H & E, × 530.
Fig. 4. Histologic type C, atrophy in a spontaneously regressing tumor. H & E, × 425.
Fig. 5. Histologic type D, secretion-like activity in well-differentiated glands. H & E, × 425.
Fig. 6. Variant of type D with a trabecular pattern. H & E, × 425.
Fig. 7. Alkaline phosphatase reaction in myoepithelial cells of tumor. × 270.
Fig. 8. β-Glucuronidase. Many tumor cells show positive reaction, but no consistent pattern is evident. × 425.
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