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Effect of Ovariectomy on Serially Transplanted Rat Mammary Tumors Induced by 7,12-Dimethylbenz[*a*]anthracene*

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Abstract—*Mammary tumors in inbred Fischer* 344 *female rats were induced by* 7, 12-*dimethylbenz*[*a*]*anthracene* (*DMBA*). *Primary tumors were excised and transplanted subcutaneously to syngeneic recipients. The transplanted tumors, thus developed, were used for further serial transplantation of up to five passages. Tumors serially transplanted into intact recipients were able to grow to* 1.5–2 cm *in diameter within* 1–2 months. *Histologically, tumor tissues of early passages were characterized by mixed populations of epithelial and spindle cells while tumors of late passages contained only the spindle cells. Tumors grafted into ovariectomized hosts were not able to grow in the first and second passages, although they remained viable, since estrogen therapy was able to activate their growth. By the third passage, tumors were able to grow when transplanted into ovariectomized recipients but the growth rate was considerably slower than that in the intact hosts. In the fourth and fifth passages, tumors transplanted in ovariectomized recipients grew as well as those in intact hosts. Tumors grown under these hormone deficient conditions consisted mainly of spindle cells. Following the establishment of the transplanted tumors, ovariectomy in the hosts resulted in an initial regression and subsequent regrowth of tumors of early passages and had no effect on those of late passages. Again, the long-term growth of transplanted tumors in ovariectomized hosts was associated with an increase in the spindle cell population which gradually replaced the regressed epithelial cells. Contents of estrogen receptors were high in the primary tumors, averaging* 45 ± 9 fmol/mg *protein* (*mean* $\pm$ *S.E.M.*) *and were low in the late passages, being* 5.2 ± 0.7 fmol/mg. *Activities of acid ribonuclease were high in primary tumors* (1132 ± 163 units/mg *protein*), *decreased in tumors of the fifth passage* (387 ± 51 units/mg *protein*) *and reached the lowest value in tumors of the first passage following regrowth in ovariectomized hosts* (301 ± 50) units/mg *protein*). *Results suggest that the so-called 'hormone-dependent' DMBA-induced mammary tumors consist of at least two histologically and biochemically distinct cell types, hormone-dependent epithelial and hormone-independent spindle-shaped cells, and that the latter are selected over the epithelial component during serial transplantations or under unfavourable hormonal conditions due to ovariectomy in the hosts.*

INTRODUCTION

RAT MAMMARY tumors induced by DMBA are often used as an experimental model for hormone-dependent tumors [1, 2]. However, under certain circumstances, such as prolonged survival in the primary hosts [3] or repeated transplantation into syngeneic recipients [4, 5], these tumors have been described as becoming hormone-independent. This was based on the observation that the hormone-dependent tumors contain mainly epithelial cells and that the independent tumors contain mainly spindle-shaped cells. Thus, this tumor system provides an opportunity for studying the biological interactions

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of two cell types in a tumor tissue. Furthermore, although the natural history of tumor progression from hormone dependence to autonomy for pregnancy-dependent mammary tumors in GR mice [6–8] and for mammotropin-dependent MT-W9 mammary tumors in W/Fu rats [9] has been well characterized, such information for DMBA-induced mammary tumors in rats remains fragmentary [3–5].

The aim of the present work was to study the effect of ovariectomy on the course of this change from epithelial type to spindle cell type during transplantation of DMBA-induced mammary tumors in syngeneic rats. Ovariectomies were performed under the following two conditions; the first condition was to perform ovariectomy simultaneously with the implantation of tumor grafts for determining if the tumor will take and grow in the hormone deficient hosts as well as those in recipients without surgery; the second condition was to perform the ovariectomy in the tumor-bearing animals after establishment of the tumor graft for determining if the established tumor will continue to grow or will regress in a hormonal deprived host. In addition to morphological changes, the functional changes in the malignant tissues were assessed by levels of estrogen receptors and activities of acid ribonuclease in the transplanted tumor tissue.

MATERIALS AND METHODS

Fischer 344 inbred female rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). At 50 days of age, each rat was fed 10 mg of DMBA (Sigma Chemical Co., St. Louis, MO) in 0.5 ml of sesame oil (Fisher Scientific Co., Pittsburgh, PA). Mammary tumors developed in 15 out of 50 animals between 30 and 60 days after DBMA feeding and these tumors were used in this experiment. The tumor size was measured weekly by a caliper in two dimensions. Tumor volume was calculated by the formula: $(4/3)\pi ab^2$, where a = major radius and b = minor radius. Tumors used for transplantation were always removed from intact hosts. When a tumor reached 1.5–2 cm in diameter it was surgically removed from the host, placed in medium 199 (GIBCO, Grand Island, NY), and cut into small cubes of 1–2 mm^3. Four to six pieces of the cut tumor tissue were transplanted subcutaneously by a trocar along the mammary line of syngeneic recipients, sometimes in multiple sites, as previously described [10]. Recipient animals were 90-day-old females. They were either intact or ovariectomized at the time of tumor transplantation. When established grafts grew to 1.5–2 cm in diameter (or 2–4 cm^3 in volume), they were either serially transplanted up to the fifth passage or followed for the pattern of regression (or growth) after their hosts were ovariectomized (or sham-operated).

Changes in tumor volume as a result of ovariectomy (or sham operation) were expressed as a percent of the original volume. Individual tumors were surgically removed for use in transplantation and/or at monthly intervals for histologic and biochemical examination. For histologic examination one portion of tissue from each tumor was fixed in 10% buffered formalin, embedded in paraffin, cut 6 μm thick, and stained with H&E. The remainder of the tumor was immediately stored in liquid nitrogen for later determination of estrogen receptors and acid ribonuclease.

Estrogen receptors in the tumor cytosol were measured by a charcoal absorption method with the use of the principle of Scatchard [11, 12] and were expressed in fmol/mg protein as described previously [13]. The activity of acid ribonuclease in the tumor tissue was determined according to the procedure of Takahashi [14] using purified soluble yeast RNA as the substrate and was expressed in units/mg protein [15]. The protein content of tissue cytosol was measured by the method of Lowry *et al.* [16].

All statistical data were indicated by mean $\pm$ standard error. Analysis of variance test and Duncan's multiple range test [17] were employed to compare differences among mean values of different treatment groups.

RESULTS

Figure 1 summarizes the result of five serial transplantations of mammary tumors into intact and ovariectomized syngeneic recipients. Ovariectomy in these animals was performed at the same time as tumor transplantation. The tumor size, indicated as cm^3, is the average of 20 tumors, originating from four primary tumors with five recipients at each passage. One tumor failed to establish during the first passage and another did not grow during the second passage. These two tumors were not included in the present result. The number of days indicated with each passage in Fig. 1 is the interval between tumor grafting and the time that the average tumor size

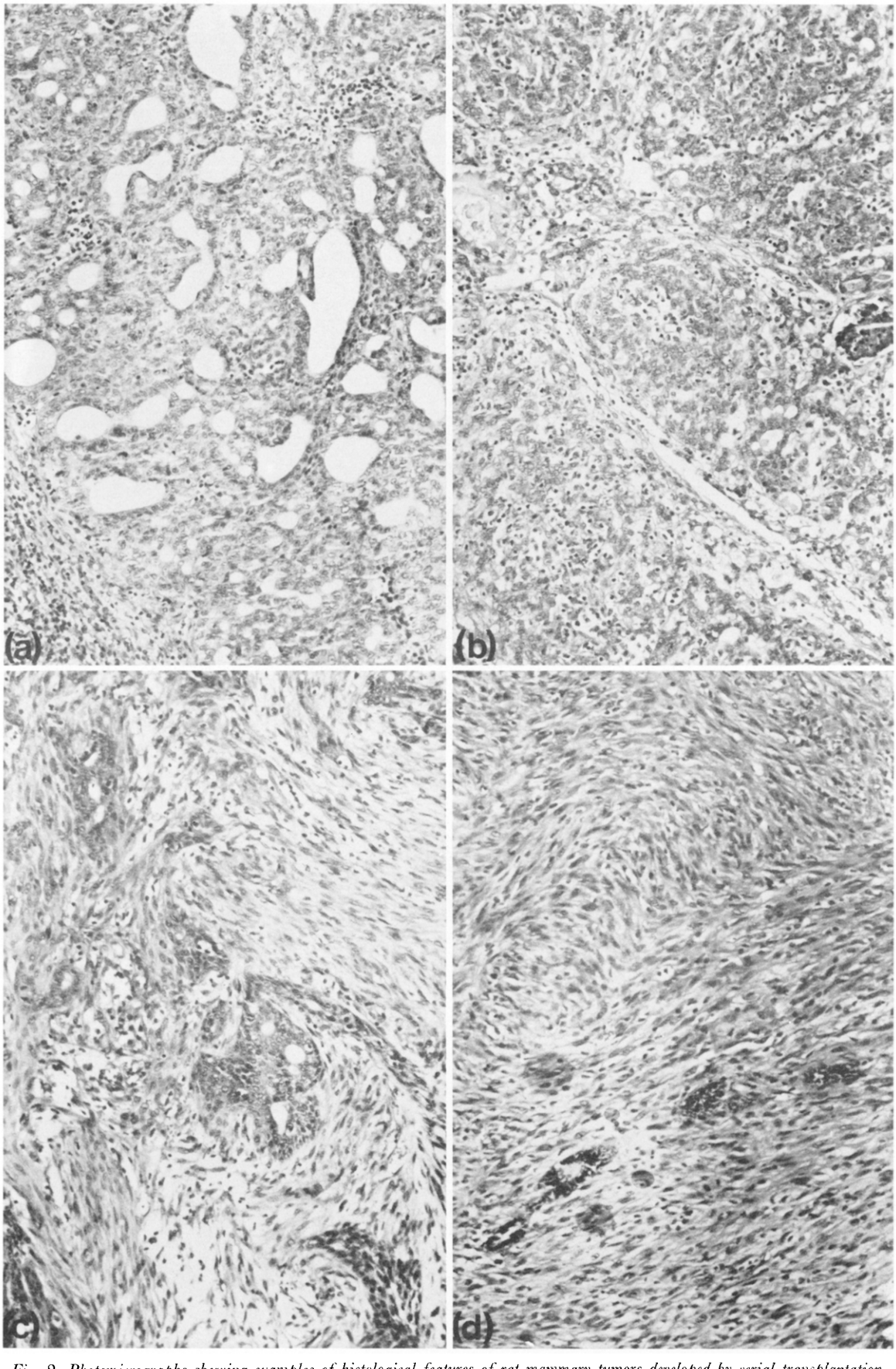

Fig. 2. Photomicrographs showing examples of histological features of rat mammary tumors developed by serial transplantation. H&E, ×40. (*A*) *A primary tumor. Epithelial cells form large nests within which cribriform pattern of ductal adenocarcinoma is evident. Stromal cells with non-malignant cytological features are in the left lower corner. The stroma is sprinkled with a small number of lymphocytes and macrophages.* (*B*) *A tumor of the first passage. Epithelial nests are well maintained and are not different from those of primary tumors, although some areas are suggestive of having increased stromal components.* (*C*) *A tumor of the third passage. Small epithelial nests scattering among spindle-cell masses are commonly seen in the tumor.* (*D*) *A tumor of the fifth passage. Densely cellular tumor is composed of spindle-cells in bundles and whirls. The tumor contains few small islets of epithelial cells. Lymphocytic infiltration is very sparse.*

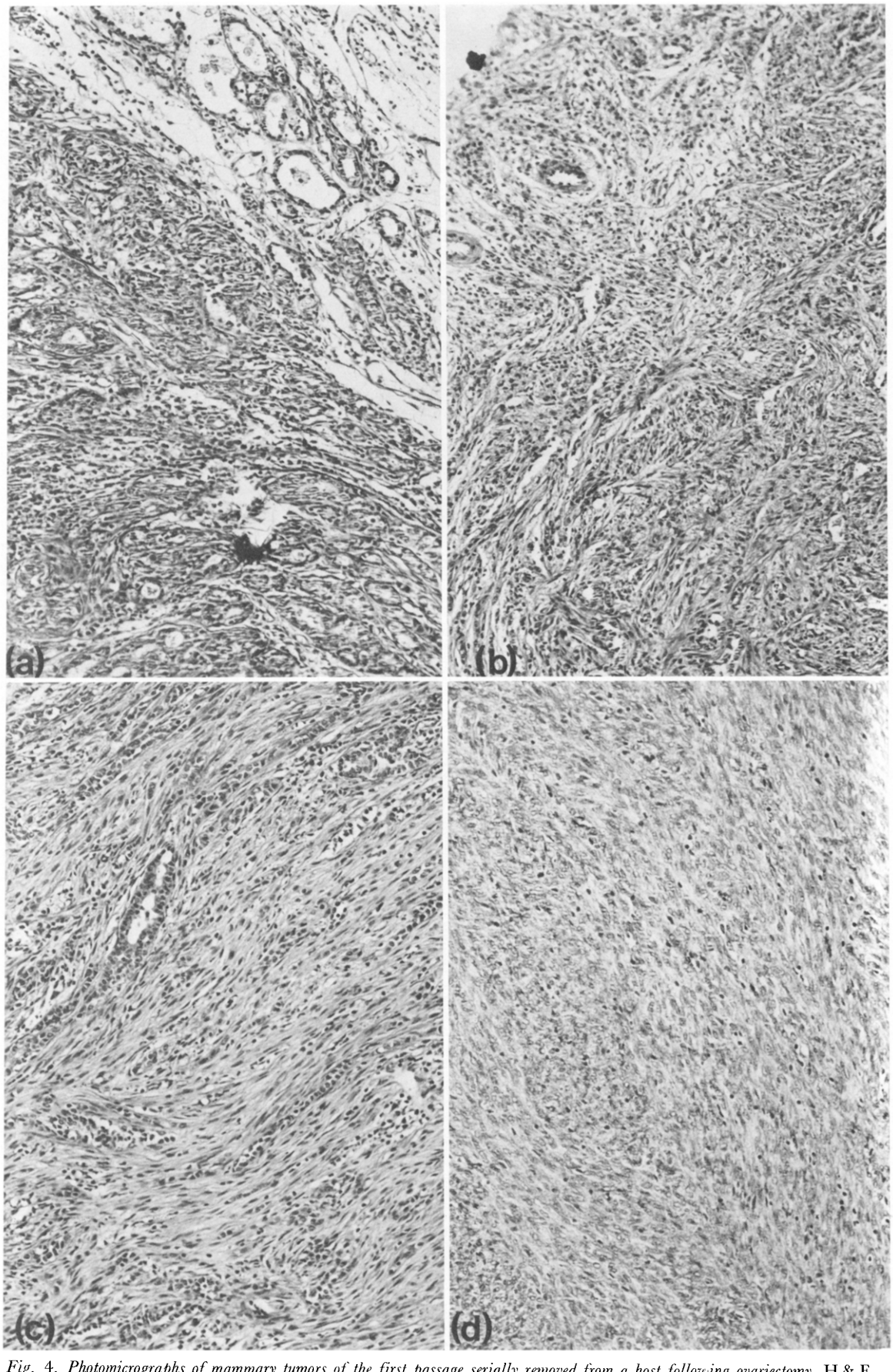

Fig. 4. *Photomicrographs of mammary tumors of the first passage serially removed from a host following ovariectomy.* H & E, ×40. (A) 2 days *after ovariectomy*, (B) 2 weeks *after ovariectomy*, (C) 1 month *after ovariectomy*, (D) 3 months *after ovariectomy*.

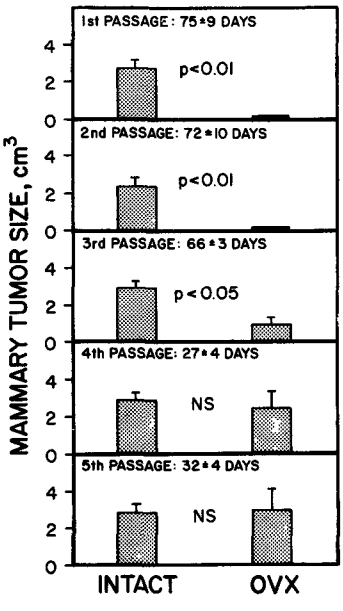

Fig. 1. Patterns of growth of rat mammary tumors serially transplanted into intact and ovariectomized animals. The procedure of transplantation is described in Materials and Methods. Each bar represents the average tumor size from 20 animals at each passage. The vertical line denotes a standard error. The number of days listed for each passage indicates the average interval from the day of transplantation to the day when the tumors reached the reported size.

reached the recorded volume. Since the average tumor size for the intact recipients was similar among passages, this interval is thus taken as the 'latent period' for the average grafts to grow to 2.4–2.9 cm^3. In the intact recipients, this latent period for the first three passages was significantly longer ($P<0.01$) than those of the fourth or fifth passages. Histologic examination of transplanted tumors in intact host revealed a gradual change from the structure of a well differentiated adenocarcinoma [18] to that of a fibrosarcoma-like spindle-cell tumor (Fig. 2).

Effects of ovariectomy on new grafts

When grafts of the first two passages were transplanted into animals in which ovariectomy was performed simultaneously, they did not develop into palpable tumors. Such implants, however, did not regress completely, and they remained viable in this dormant state for at least 2 months. The growth of these dormant grafts could be activated with a supply of estrogens by subcutaneously implanted silastic tubes containing 0.5 cm of estradiol [19]. This procedure provided the maintenance of a constant serum estradiol level of about 75 pg/ml. Tumor growth would begin again after 7 days. Such tumors had the pattern of adenocarcinoma. All grafts of the third passage grew in ovariectomized recipients as well as in intact rats. However, the growth of grafts of the third passage in ovariectomized recipients was delayed and the average tumor size was significantly smaller ($P<0.05$) than that in the intact recipients during the same latent period (Fig. 1). In the fourth and fifth passages, grafts grew equally well in intact and ovariectomized recipients. All tumors grown in ovariectomized hosts showed the histological features of spindle-cell tumors.

Effects of ovariectomy on established tumors

Figure 3 shows the pattern of growth (or regression) of established tumors after their host animals were ovariectomized or sham-operated. Ovariectomy in rats bearing primary tumors resulted in regression of the tumors. However, these regressed tumors remained dormant, because estrogen replacement by implanting silastic tubes containing estradiol reactivated tumor growth. The histology of these reactivated tumors had the features of adenocarcinoma.

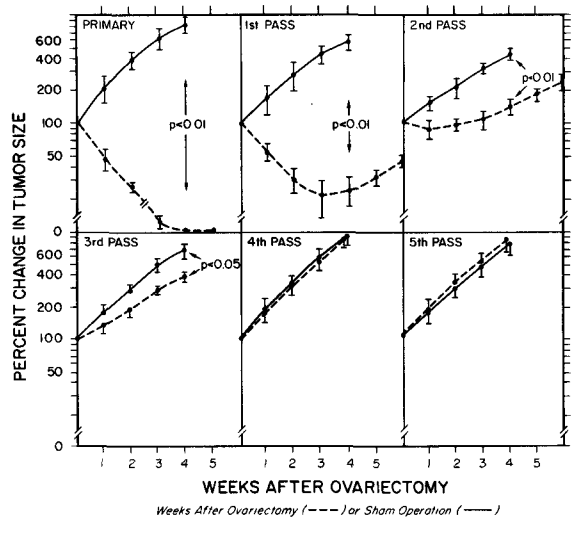

Fig. 3. Effect of ovariectomy or sham operation on growth (or regression) of established mammary tumors at different generations of transplantation. Each point represents the average of 6–10 individual tumors removed serially from animals bearing multiple transplanted tumors. The vertical line denotes a standard error. P *value is the statistical significance between the two groups compared at the time indicated by the arrows.*

By contrast, established tumors of the first passage underwent an initial regression following ovariectomy in their hosts, but they relapsed without further treatment during the next 3–4 weeks (Fig. 3). Histologic studies indicated that tumors which reappeared after ovariectomy were characterized by spindle cells (Fig. 4). Ovariectomy had no effect on established tumors of the second and third

passages but the growth of these tumors was significantly delayed (Fig. 3). Histologic studies revealed again that these tumors were constituted by spindle-cells. In the fourth and fifth passages, there was no difference in growth rate of tumors between the intact and ovariectomized hosts. Pulmonary metastases were found in two animals bearing tumors of the fourth passages.

Table 1. Estrogen receptors in rat mammary tumors at different passages of transplantation

Number of passages	Number of tumors	Levels of estrogen receptors (fmol/mg protein)	Association Constant ($\times 10^9 M^{-1}$)
Primary	7	45.3±9.0*	2.57±0.28
First	8	30.4±3.0†	2.09±0.29
Second	8	22.5±3.0‡	2.25±0.37
Third	8	15.7±2.0	2.71±0.42
Fourth	6	4.8±1.1	2.55±0.55
Fifth	7	5.2±0.7	2.67±0.63

Results expressed as mean±S.E.
*The value is significantly greater than values of other passages ($P<0.01$).
†The value is significantly greater than values of third ($P<0.05$), fourth and fifth ($P<0.01$) passages.
‡The value is significantly greater than values of fourth and fifth passages ($P<0.01$).

Table 1 lists the levels of cytosol estrogen receptors in mammary tumors of various passages. There is a significant decrease ($P<0.01$) in estrogen receptors of the mammary tumors as the generation of tumor transplantation advances. Contents of estrogen receptors were high in the primary tumors, averaging 45±9.0 fmol/mg protein, and were low in the late passages, being 5.2±0.7 fmol/mg. As mammary tumors advanced through serial transplantation, activities of acid ribonuclease reduced significantly ($P<0.01$; Table 2). Furthermore, long-term growing tumors removed from the ovariectomized hosts all contained low activities of acid ribonuclease (301±50 units/mg protein) similar to those observed in the tumors of fourth and fifth passages in the intact hosts.

DISCUSSION

Results of the present study indicate that DMBA-induced hormone-dependent mammary adenocarcinoma in rats can be converted to hormone less dependent or independent tumors by transplantation into syngeneic hosts or by ovariectomy in the host animals. This observation agrees with the earlier findings of others [4, 5]. In the primary tumor first and second passages, dormancy of the transplanted tissue in ovariectomized rats, which can be reversed in an appreciable time by administration of exogenous estradiol, demonstrates the functional dependence of the developing tumor on the hormonal conditions. In this study, we have further demonstrated the subsequent transitional stages of the conversion. In addition,

Table 2. Acid ribonuclease in rat mammary tumors of different passages of transplantation

	Tumors removed from intact hosts		Tumors removed from ovariectomized hosts	
Number of passages	Number of tumors	Enzyme activity (units/mg protein)	Number of tumors	Enzyme activity (units/mg protein)
Primary	8	1133±162*	—	—
First	8	730±38†	4	300±22‡
Second	8	591±54	4	409±45
Third	8	509±23	5	350±36
Fourth	8	426±24	4	355±38
Fifth	8	382±31	4	353±52

Results expressed as mean±S.E.
*The value is significantly greater than values of other passages ($P<0.01$).
†The value is significantly greater than values of fourth and fifth passages ($P<0.01$).
‡The value is significantly smaller than that of tumors of the corresponding passage of intact hosts ($P<0.01$).

we have learned that these morphologic changes are accompanied by a concomitant reduction in content of estrogen receptors and in activities of acid ribonuclease in the tumoral tissue.

The present data suggest that serial transplantation of DMBA-induced mammary tumors or ovariectomy facilitates a cellular selection process [20]. Referring to the work of Hauschka [21] and Klein and Klein [22], Heppner [23] pointed out that the capacity of experimental tumors to show discrete qualitative and quantitative changes over the course of serial transplantation was the outcome of the selection of variants present within the tumor cell population. Our results and those of Griswold and Green [3] further indicate that the endocrine interference is of importance for selection of hormone-independent spindle cells since such selection could also be achieved by ovariectomy, i.e., by maintaining the tumor under hormonal conditions unfavorable for the survival of hormone-dependent epithelial cells. Because of the presently observed phenomenon, if it is true that ovariectomy facilitates the development of hormone-independent tumor cells, the current procedures of endocrine therapy in patients with estrogen receptor-positive or hormone-responsive breast cancer perhaps should be evaluated more critically.

The results of the present study seem to indicate that the natural history of progression from hormone-dependent tumors with high estrogen receptor content to autonomous tumors with low estrogen receptor content occurs in DMBA-induced rat mammary tumors. This course of progression is similar to those of mammatropin-dependent MT-W9 mammary tumors in rats [9] and of pregnancy-dependent mammary tumors in GR mice [6–8]. In all of these cases, when hormone-responsive tumors were grafted into ovariectomized recipients, they gave rise to hormone-independent outgrowths. Sluyser explained that, in the absence of trophic hormones, only the autonomous cells in the graft could proliferate and that these yielded an autonomous outgrowth [8]. Sluyser continued to say that during serial transplantation there is an increase in the percentage of autonomous relative to hormone-dependent cells in the tumors. The main reason probably was not that hormone-independent cells multiply more rapidly, but rather that fewer independent cells than hormone-dependent cells were required in order for a graft to take. Furthermore, during growth of the tumor the autonomous cells were better adapted to the tumor environment and, therefore, were selected out [8].

If the above explanation applies to all mammary tumors, including human breast cancers, those containing large amounts of hormone-dependent cells should be considered as being in the early stages of tumor progression and those with predominant autonomous cells should be considered as advanced tumors. Results of our present study on DMBA-induced tumors and those of others on MT-9W rat tumors and tumors in GR mice seem to support this concept.

The evidence of two cell populations and the selection from one cell type to another is further supported by the changing levels of estrogen receptors and acid ribonuclease in tumors of various passages. Our data are consistent with the assumption that epithelial cells possess high levels of both estrogen receptors and ribonuclease, and that spindle-cell tissue contains very little of both. The development of spindle-cell tissue in this tumor system must, however, be viewed with caution. It is as yet uncertain whether the appearance of spindle-cell tumors was the result of a morphologic transformation from the epithelial stem cells [24, 25] or was derived from a separate cellular source of stromal origin [26]. Characterizing the origin of these spindle-cell tissues, therefore, warrants further study.

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