

Detection of Ductal Dysplasia in Mammary Outgrowths Derived from Carcinogen-treated Virgin Female BALB/c Mice¹

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ABSTRACT

These studies were undertaken to determine if altered growth potential of mammary epithelial cells could be detected in outgrowths derived from monodispersed mammary cells of virgin female BALB/c mice previously exposed to ionizing radiation or 7,12-dimethylbenz(a)anthracene (DMBA).

Monodispersed mammary epithelial cells were obtained by enzymatic dissociation of mammary tissues of 12-week-old virgin female BALB/c mice. Twenty-four hr prior to cell dissociation, donor animals were exposed to either 100 rads of γ -ray irradiation, 0.25 mg of DMBA, or 0.075 mg of DMBA. Control donors were untreated. Mammary outgrowths were then derived from these donor cells by injecting either 10^5 or 10^4 cells into the gland-free mammary fat pads of three-week-old virgin female BALB/c mice. Ten weeks after the injection of cells, the outgrowths were examined and classified. Mammary outgrowths were classified either as having a normal ductal architecture or as having ductal dysplasia. Ductal dysplasias were further classified on the basis of an index of severity, which was an arbitrary index based on the number of abnormal ductal structures within each lesion. The data indicated that treatment of donor animals with either γ -radiation or DMBA increased the frequency of ductal lesions over control levels; however, both the frequency and severity of the lesions depended on the number of cells which were injected into the fat pad. When outgrowths were derived by injection of 10^5 cells into the gland-free fat pads, lesion frequencies in outgrowths from control and treated cells were: 3.3%, control; 15.7%, γ -rays; 5.3%, 0.25 mg DMBA; in these groups only a few severe lesions were detected. In outgrowths derived from 10^4 cells, less severe lesions (Class I lesions) were common in all groups and occurred in approximately 10 to 15% of the outgrowths. The frequency of severe (Class II and III) ductal dysplasia, however, was increased by treatment in these groups, occurring in 4.5% of control outgrowths in 15.6, 14.9, and 14.3% of the outgrowths derived from donor cells treated with 100 rads γ -rays, 0.075 mg DMBA, and 0.25 mg DMBA, respectively. Thus, these data indicated that ductal dysplasias were more common and more severe in outgrowths derived from 10^4 rather than 10^5 cells. The ductal lesions observed in this study resembled both morphologically and histologically ductal abnormalities which have been associated with the pathogenesis of mammary carcinoma in both rats and mice.

INTRODUCTION

Mammary tumors which arise in rodents, whether by the action of chemical carcinogens or the MuMTV,³ are usually preceded by discrete lesions which have high neoplastic potential relative to normal tissue (15, 17). One such lesion is the HAN. HAN can be induced in both rats (3, 27) and mice (9) by administration of chemical carcinogens and by the MuMTV in mice (7). Transplantation studies have indicated that this lesion has tumor potential greater than that of normal tissue in both species, independent of the mode of induction (2, 4, 5, 16, 22). DeOme *et al.* (6) have provided evidence which suggests that the expression of nodule-transformed cells as HAN in mice bearing the MuMTV is modified by the presence and architecture of normal mammary cells. This hypothesis stems from the observation that, when mammary cells from mice with MuMTV expressed were enzymatically dissociated and transplanted into the gland-free fat pads of syngeneic hosts, the expression of HAN was markedly enhanced in the outgrowths which resulted. Further support for this hypothesis came from Medina *et al.* (18), who showed that the tumor potential of HAN-derived outgrowth lines could be enhanced by enzymatic dissociation and transplantation of these cells into the gland-free fat pads of virgin hosts.

In the rat, the ductal origin of mammary carcinoma has been well established (20, 23, 24), and there is a good deal of evidence which indicates that these tumors are preceded by ductal dysplasias (10, 14, 21). Mammary lesions of ductal origin have also been observed in mice with MuMTV unexpressed following treatment with DMBA, and although in the past less emphasis has been placed on the importance of these lesions in mouse mammary tumorigenesis, evidence is available which indicates that these lesions also have high neoplastic potential (17). Recent reports have suggested that the cell dissociation assay can be used to detect ductal mammary lesions induced by exposure of mice with MuMTV unexpressed to DMBA (8, 11) or to ionizing radiation (8).

In this report, we present the results of our experiments which indicated that altered growth potential of mammary epithelial cells induced by exposure of donor mice to chemical and physical carcinogens resulted in the expression of mammary ductal dysplasia which could be detected using the cell dissociation technique. In addition, we report evidence which indicated that the expression of ductal dysplasias was influenced by the number of donor cells which were transplanted into the host fat pads.

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³ The abbreviations used are: MuMTV, murine mammary tumor virus; HAN, hyperplastic alveolar nodule(s); DMBA, 7,12-dimethylbenz(a)anthracene.

MATERIALS AND METHODS

Animals. In these experiments, germ-free-derived, specific-pathogen-free virgin female BALB/cAnNBd mice, which were bred and maintained in the Biology Division barrier facility, were used. The animals were housed 8/cage and were fed Purina laboratory chow and water *ad libitum*.

Carcinogen Exposure. Donor mice were exposed to either γ -ray irradiation or DMBA when they were 12 weeks old. DMBA (Sigma Chemical Co., St. Louis, Mo.) was dissolved in stripped corn oil (Eastman Kodak Co., Rochester, N. Y.) and administered to unanesthetized mice by gastric intubation via a 20-gauge needle ending in a silver-soldered bulb. γ -Ray irradiation was carried out with a 2000-Ci ^{137}Cs source at a dose rate of 32.8 rads/min. During the irradiation, the animals were kept in plastic tubes and rotated in the beam. Details of the irradiation procedures have been published (28). Control donors were either sham irradiated or untreated.

Experimental Protocol. Twenty-four hr after the administration of carcinogen, donor mice were anesthetized with Diabulal (Diamond Laboratories, Inc., Des Moines, Iowa), and the mammary tissues were exposed via a midventral incision. The mammary tissues were then removed free of lymph nodes and other extraneous tissues. The mammary tissues were weighed and minced with razor blades on an inverted glass Petri dish. The minced tissues were then suspended in an enzyme preparation consisting of 0.1% collagenase (107 units/mg; Millipore Corp., Freehold, N. J.) and 0.1% hyaluronidase (255 units/mg; Sigma) at a concentration of 20 ml/g of tissue and incubated at 37° in a shaking water bath for 90 min. Next, the cells were centrifuged, and the pellet and fatty layer was transferred to a second enzyme preparation consisting of 0.5% Pronase (56,300 proteolytic units Koken/g; Calbiochem., La Jolla, Calif.) and incubated at 37° in the shaking water bath for 30 min. The resulting cell suspension was centrifuged and washed 3 times in fresh McCoy's media, filtered through 20- μm Nitex (Tetko, Inc., Elford, N. Y.), and counted on a hemocytometer. Cell viability was determined by trypan blue exclusion. The cells were then centrifuged and resuspended so as to have either 10^4 or 10^5 viable cells in 0.01 ml of media.

Host animals were 3-week-old virgin female BALB/c mice. The inguinal mammary glands of the host mice were cleared of the mammary rudiments by the method of DeOme *et al.* (5). Then either 10^5 or 10^4 viable cells were injected into the gland-free fat pads of the host mice using a 27-gauge needle attached to a Hamilton syringe (Hamilton Co., Reno, Nev.). When inocula of 10^5 cells were transplanted, 32 fat pads per donor mouse were treated with cells. When inocula of 10^4 cells were used, 48 fat pads per donor mouse were treated with cells.

Ten weeks after the injection of cells, the host animals were killed and both fat pads were removed, along with a piece of the third mammary gland. The tissues were spread on Tissue Tek capsules and fixed in 100% ethanol:glacial acetic acid (3:1) for 24 hr. Following fixation, the tissues were stained with alum carmine stain according to the method described by Banerjee *et al.* (1).

For histological examination of tissues, stained mammary tissues were either embedded in paraffin or methacrylate. Five- μm sections were cut from paraffin-mounted tissues and stained with hematoxylin and eosin, while from the methacrylate-mounted tissues, 1- μm sections were cut and stained with methylene blue and basic fuchsin.

Classification of Outgrowths. The mammary outgrowths that were derived by the method just described were classified as having a normal ductal architecture or having ductal dysplasia. Outgrowths were classified only if the origins of the outgrowths could be seen to arise in the center of the fat pad, thus ensuring that the outgrowths arose from injected cells and not from remnants of host mammary tissue. The normal ductal outgrowths resembled the host mammary glands and were characterized by ducts that branched at regular intervals and maintained a uniform spacing. Normal ducts terminated by sending off 2 or 3 terminal ducts which were capped by end buds if the duct terminus had not reached the edge of the fat pad (Fig. 1). Outgrowths

with ductal abnormalities most often exhibited abnormal terminal structures. These abnormal termini consisted of several (up to 10 and sometimes more) terminal ducts which were capped by hyperplastic end buds. These end buds were lined by a thickened epithelium which protruded into and often occluded the lumen. Hyperplastic end buds were also occasionally observed in outgrowths which were without abnormal termini. In these outgrowths, the branching pattern was irregular, and hyperplastic end buds could be seen at the end of side branches. Although nearly all of the abnormal outgrowths that were observed could be classified on the basis just described, there was a good deal of variability in the number of abnormal terminal structures and hyperplastic end buds observed within the abnormal outgrowths in all the groups that were examined. For this reason, the ductal lesions were further classified by constructing an index of severity. This index was an arbitrary scale and was based upon (a) the number of abnormal termini within a given outgrowth; (b) the number of hyperplastic end buds which were associated with the abnormal terminus; and (c) the fraction of the end buds on an abnormal terminus which appeared hyperplastic. Lesions which were without abnormal termini were classified on the basis of the number of hyperplastic end buds in the outgrowth. Using this index, mammary lesions were categorized into 3 groups. Class I lesions had no more than one and often were without abnormal terminal structures, but had ducts which were capped by a few hyperplastic end buds (Fig. 2). Class II lesions were those which had at least one abnormal terminus consisting of terminal ducts capped by several hyperplastic end buds (Fig. 3). Class III lesions were those which consisted of several abnormal terminal structures, each of which had terminal ducts capped by hyperplastic end buds (Fig. 4).

Mammary ducts and end buds, which appeared hyperplastic at the whole mount level, were characterized at the histological level by several layers of epithelial cells that formed chords of cells which protruded into, and often occluded, the ductal lumina. The epithelial cells themselves were irregular in size and shape, and mitotic figures were frequent (Figs. 5 and 6).

RESULTS

In these experiments, mammary outgrowths were derived by injection of either 10^5 or 10^4 monodispersed mammary cells into the gland-free fat pads of 3-week-old virgin female BALB/c mice. These cells gave rise to outgrowths in 80 to 90% of the injected rat pads, and this was not affected by exposure of donor animals to either radiation or DMBA (Table 1). In outgrowths derived from 10^4 cells, carcinogen exposure did decrease slightly the number of outgrowths which filled or nearly filled the rat pads by 10 weeks after injection of cells. Outgrowths exhibited for the most part a normal ductal architecture; however, some outgrowths with abnormal morphology

Table 1
Cell dissociation-derived outgrowths from control and carcinogen-treated virgin female BALB/c mice

Treatment	Cell dose	Classifiable outgrowths (%)	Classifiable outgrowths which filled at least $\frac{3}{4}$ of the fat pad (%)
Control	10^5	83.3 (120/144) ^a	94.4 (113/120) ^b
γ -irradiation (100 rads)	10^5	84.4 (108/128)	85.4 (92/108)
DMBA (0.25 mg)	10^5	79.2 (76/96)	90.9 (69/76)
Control	10^4	83.5 (132/158)	80.3 (106/132)
γ -irradiation (100 rads)	10^4	83.7 (77/92)	57.1 (44/77)
DMBA (0.25 mg)	10^4	87.5 (84/96)	71.4 (60/84)
DMBA (0.075 mg)	10^4	90.6 (87/96)	67.8 (59/87)

^a Numbers in parentheses, number of outgrowths with sufficient ductal growth to be classified as normal or abnormal/total number of fat pads injected.

^b Numbers in parentheses, number of outgrowths which filled at least three-fourths of the fat pad by 10 weeks after injection of cells/total number of classifiable outgrowths.

Table 2

Occurrence of mammary ductal dysplasias in outgrowths derived from either 10^5 or 10^4 monodispersed mammary cells of virgin female BALB/c mice which were exposed to either γ -ray irradiation or DMBA 24 hr prior to cell dissociation

Treatment	Cell dose	Positive donors	Frequency of mammary ductal dysplasias (%)			
			Class I	Class II	Class III	Class II + III ^a
Controls	10^5	2/7 ^b	2.5 \pm 1.4 (3/120) ^c	0.8 \pm 0.8 (1/120)	0	0.8 \pm 0.8 (1/120)
γ -irradiation (100 rads)	10^5	3/4	10.2 \pm 2.9 (11/108) ^d	4.6 \pm 2.0 (5/108) ^e	1.8 \pm 1.3 (2/108)	6.4 \pm 2.3 (7/108) ^d
DMBA (0.25 mg)	10^5	2/4	5.3 \pm 2.6 (4/76) ^f	0	0	0
Controls	10^4	4/4	14.4 \pm 3.1 (19/132)	1.5 \pm 1.1 (2/132)	3.0 \pm 1.5 (4/132)	4.5 \pm 1.8 (6/132) ^d
γ -irradiation (100 rads)	10^4	2/2	9.1 \pm 3.3 (7/77) ^f	6.5 \pm 2.8 (5/77) ^e	9.1 \pm 3.3 (7/77) ^e	15.6 \pm 4.1 (12/77) ^d
DMBA (0.25 mg)	10^4	2/2	14.3 \pm 3.8 (12/84) ^f	7.2 \pm 2.8 (6/84) ^e	7.2 \pm 2.8 (6/84) ^e	14.3 \pm 3.8 (12/84) ^d
DMBA (0.075 mg)	10^4	2/2	9.2 \pm 3.1 (8/87) ^f	3.4 \pm 1.9 (3/87) ^f	11.5 \pm 3.4 (10/87) ^d	14.9 \pm 3.8 (13/87) ^d

^a Data for the frequency of Class II plus Class III lesions are given because in 10^4 cell-derived outgrowths the frequencies of Class I lesions were not affected by treatment.

^b Number of donors whose cells yielded lesions/all donors used in the experimental group.

^c Numbers in parentheses, number of outgrowths with lesions/number of classifiable outgrowths in the group.

^d $p < 0.05$ versus control.

^e $p < 0.1$ versus control.

^f Not significantly different from controls.

were detected, and these were classified as ductal dysplasias. (Mammary lesions of any kind have not been detected in the intact mammary glands of 3-month-old to 12-month-old virgin female BALB/c mice.)

Detection of Ductal Dysplasias. Mammary ductal dysplasias were detected in outgrowths derived from the cells of both treated and nontreated donor animals. The frequency of occurrence, as well as the severity of these lesions, was influenced by carcinogen treatment and by the number of donor cells which were injected into the host fat pads.

The outgrowths which were derived from 10^5 control cells exhibited a low frequency (3.3%) of ductal lesions. The ductal abnormalities observed in this group were not severe; hence, all but one of these were classified as Class I lesions. A significantly higher frequency (18.9%) of ductal lesions was detected in the outgrowths derived from injection of 10^4 control cells. A comparison of the control data given in Table 2 indicates that this increase was due largely to a marked increase in the frequency of Class I lesions; however, a few severe ductal dysplasias (Class II and III) were also detected.

Exposure of donor mice to 100 rads of γ -ray irradiation prior to cell dissociation resulted in an increased frequency of ductal dysplasias over control levels, and these data are given in Table 2. This increase was detected in outgrowths derived from both 10^5 and 10^4 cells; however, the distribution of lesions within the 3 severity classes differed in the 2 groups. In the outgrowths derived from 10^5 cells, radiation exposure resulted in a 5-fold increase in the frequency of Class I and Class II lesions over control levels. In addition, a low frequency (1.8%) of Class III lesions was also detected in the outgrowths of this group. Class III lesions were not observed in 10^5 cell-derived control outgrowths. In the outgrowths derived from 10^4 cells, radiation exposure resulted in an increase in the frequency of the more severe Class II and Class III lesions, while the frequency of Class I lesions remained at control levels.

Qualitatively similar results were obtained from outgrowths derived from cells of donor animals treated with DMBA prior to cell dissociation (Table 2). After treatment with 0.25 mg of DMBA in outgrowths derived from 10^5 cells, Class I lesions were the only abnormalities detected. Although the frequency of Class I lesions was slightly elevated over the control level in this group, the difference was not significant. In outgrowths derived from 10^4 cells, treatment of donor mice with 0.25 mg of DMBA resulted in a significant increase in the frequency of

Class II and Class III lesions. In this group, as in those derived from 10^4 γ -irradiated cells, DMBA treatment did not yield an increased frequency of Class I lesions over the control level.

In order to examine the effects of a lower dose of DMBA, several donor animals were exposed to 0.075 mg of DMBA prior to cell dissociation. Outgrowths derived from 10^4 cells taken from these donors exhibited a similar increase in the incidence of Class II and Class III lesions over control levels. Here, as in the previously described groups, treatment with DMBA did not influence the frequency of Class I lesions.

DISCUSSION

In these experiments, we have detected ductal dysplasias in the mammary outgrowths derived from cells of virgin female BALB/c mice which were either untreated or were exposed to DMBA or γ -radiation.

The induction of mammary ductal dysplasias in the mammary tissues of mice with MuMTV unexpressed following exposure to DMBA has been reported previously. Medina and Warner (19) have detected these lesions in the mammary tissues of BALB/c mice 8 to 10 months after exposure to 3 or 6 mg of DMBA. Guzman *et al.* (11) have used the cell dissociation technique to detect ductal dysplasias in outgrowths derived from DMBA-treated C57BL donor cells. In Guzman's work, donor animals were exposed to 1 mg of DMBA at 5 and 6 weeks of age. Several weeks after exposure, the donor mammary tissues were removed, dissociated, and transplanted. In our work, BALB/c donor animals were exposed to low doses of either DMBA or γ -radiation at 12 weeks of age, and only 24 hr elapsed between exposure and cell dissociation. Using this protocol, we have detected ductal dysplasias in outgrowths derived from both treated and untreated donor cells. Thus, our data confirm and extend the findings of Guzman *et al.* and support the hypothesis that cell dissociation enhances the expression of ductal abnormalities induced by both chemical and physical carcinogens, as it does for the expression of nodule-transformed cells as HAN in mice with MuMTV expressed. Although our data and that of Guzman *et al.* are not directly comparable because of differences in experimental protocol, it is interesting to note that in our experiment we detected a low frequency of ductal dysplasias in outgrowths derived from control cells, while Guzman *et al.* (11) did not. This may be a reflection of differences between the BALB/c

and C57BL strains.

A significant finding of these experiments concerns the influence of cell dose on the frequency and severity of ductal dysplasias. Several observations from our experiments indicate that expression of ductal dysplasia is enhanced in both frequency and severity in outgrowths derived from 10^4 rather than 10^5 cells: (a) in the 10^4 cell-derived outgrowths, the frequencies of all lesions in each of the exposure groups were greater than those for the corresponding groups derived from 10^5 cells; (b) severe lesions (Class II and III) which were observed in control outgrowths were almost exclusively found in those derived from 10^4 cells. Indeed, ductal lesions tended to be more severe in 10^4 cell-derived outgrowths in all exposure groups; (c) exposure regimens which were ineffective in inducing lesions when outgrowths were derived from 10^5 cells (0.25 mg DMBA) induced significant numbers of Classes II and III lesions in 10^4 cell-derived outgrowths. Thus, our data indicate that detection of severe ductal dysplasia is greatly facilitated by using inocula of 10^4 cells to derive mammary outgrowths. Although the reason for the observed dependence of lesion expression on cell dose is not clear, our findings are not inconsistent with what has been observed by others. For example, DeOme *et al.* (6) and Medina *et al.* (18) have provided data which indicate that the expression of altered growth potential of nodule-transformed mammary cells is influenced by the presence of normal mammary cells. In addition, several workers have reported that expression of neoplastic transformation *in vitro* is inversely proportional to the number of cells which are seeded into culture (12, 13, 25). Further investigation will be required in order to understand the nature of the cell dose dependence which we have observed in our experiments.

The size of the inocula of donor cells also appears to have influenced the frequency of the less severe Class I lesions but in a different manner. The frequency of Class I lesions in control outgrowths derived from 10^4 cells was markedly higher than in control outgrowths derived from 10^5 cells. Further, in 10^4 cell-derived outgrowths, exposure of donor animals to radiation or DMBA did not result in an increased frequency of these lesions, as was the case with the 10^5 cell-derived outgrowths. This finding was unexpected but should be viewed with caution for the following reason. The detection of Class I lesions may have been influenced by the density of ductal growth within the mammary fat pad, which is substantially greater in the 10^5 cell-derived outgrowths than in the 10^4 cell-derived outgrowths. This difference may have allowed closer observation of individual ducts within the 10^4 cell-derived outgrowths and therefore may have facilitated detection of subtle abnormalities in the ductal architecture of these outgrowths. The similarity of the frequencies of Class I lesions in the controls and treated groups of the 10^4 cell experiment supports this view and casts some doubt on the importance of these relatively minor abnormalities. Further work, including a detailed histopathological examination of all these lesions, is in progress in order to understand better the biological significance of all of these lesion classes.

The biological significance of ductal dysplasias induced by exposure of BALB/c mice to DMBA has been studied by Medina (17). These workers have performed serial transplantation studies, the results of which indicate that ductal dysplasias have high neoplastic potential in the mouse.

The similarity of the ductal lesions which we have observed in these experiments to those which have been previously

described by Medina (16) is striking. Both are characterized at the whole-mount level by numerous and overcrowded ducts which are capped by hyperplastic end buds and at the histological level by epithelial hyperplasia which appears as papillary projections or infoldings of chords of epithelial cells into the ductal lumina. These lesions also resemble ductal dysplasias which have been observed in several rat strains following carcinogen administration (10, 21, 23). Thus, morphologically and histologically, the ductal lesions which we have found resemble ductal abnormalities for which neoplastic potential has been directly demonstrated in the mouse (17), as well as ductal dysplasias which have been associated with the pathogenesis of mammary carcinoma in the rat (10, 21, 23).

Another aspect of these data that is noteworthy concerns the fraction of exposed donors which gave rise to outgrowths which had severe lesions. When outgrowths were derived from 10^4 cells, 100% of the donors which had been exposed to DMBA or radiation had cells which yielded either Class II or Class III lesions in the outgrowths which resulted. Thus, it would appear that every animal that was exposed to these levels of carcinogen had cells with clearly altered growth potential. However, exposure of mice to the same doses results in lifetime incidences of mammary tumors of only 14% for 100 rads of γ -radiation (28) and 32 and 56% for 0.075 and 0.25 mg of DMBA, respectively.⁴ This finding is consistent with the finding of Terzaghi and Nettesheim (26), who demonstrated that preneoplastic tracheal epithelial cells with altered growth potential *in vitro* can be isolated from 100% of tracheas exposed to a dose of DMBA which yields a tumor incidence of 10%. Thus, it appears that in the mammary gland, as well as in the trachea, carcinogen treatment results in many more altered cells than are ultimately expressed as tumors.

In summary, our data indicate that exposure of virgin female BALB/c mice to low doses of DMBA or γ -radiation results in mammary epithelial cells which have altered growth potential. Furthermore, the cell dissociation assay can be used to facilitate the detection of these cells which were expressed as mammary ductal dysplasias.

Our data also indicate that these ductal abnormalities are more easily detected in outgrowths derived from 10^4 than from 10^5 cells. Finally, the ductal lesions which were observed in this study resemble ductal dysplasias for which neoplastic potential has been demonstrated experimentally, as well as presumed high-risk ductal lesions which have been associated with human breast cancer (29).

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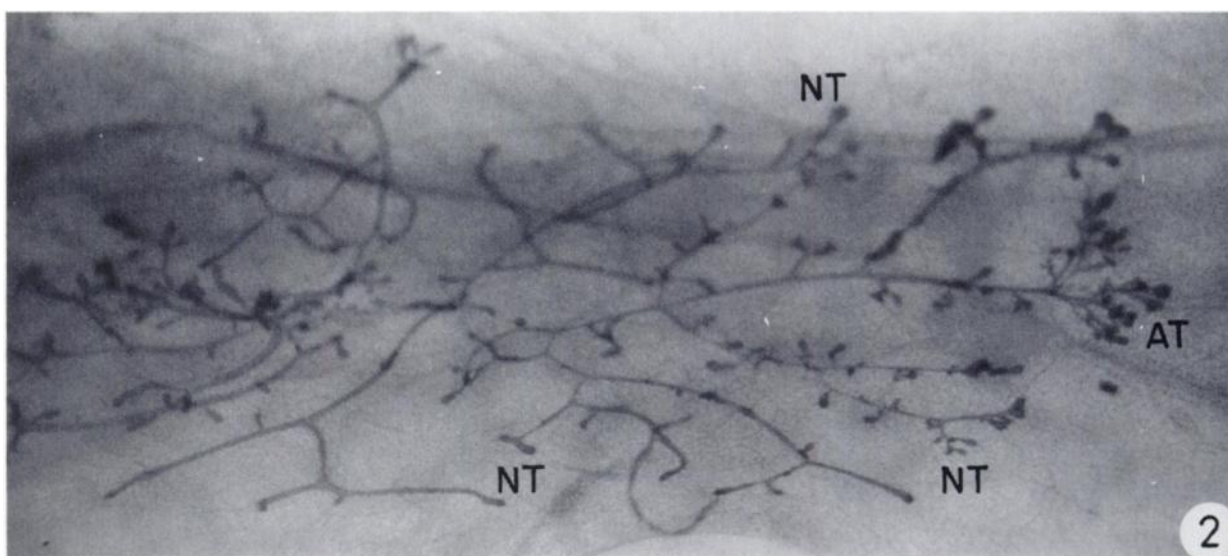
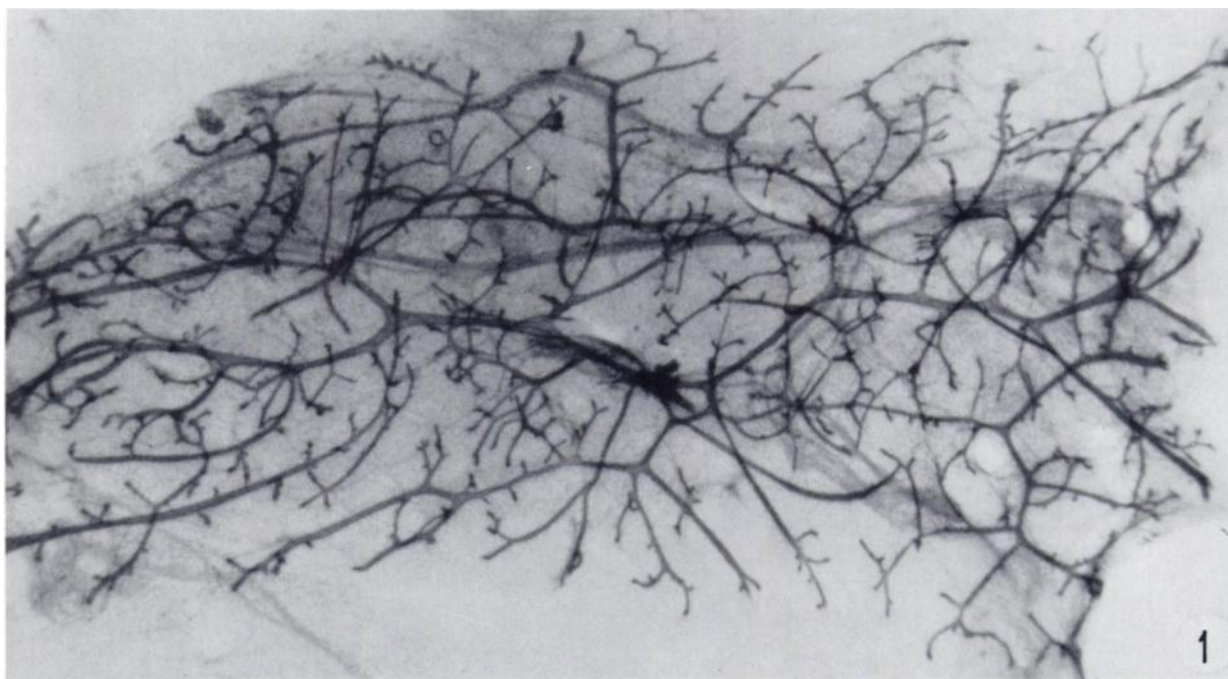


Fig. 1. Whole mount of a normal mammary outgrowth derived from 10^5 cells of a control donor. Note the dichotomous branching of ducts and duct termini. Alum carmine, $\times 20$.

Fig. 2. Whole mount of a Class I lesion derived from 10^4 cells of a control donor. The outgrowth has several normal termini (NT) with end buds and one abnormal terminal structure (AT) capped by 4 hyperplastic end buds. Alum carmine, $\times 20$.

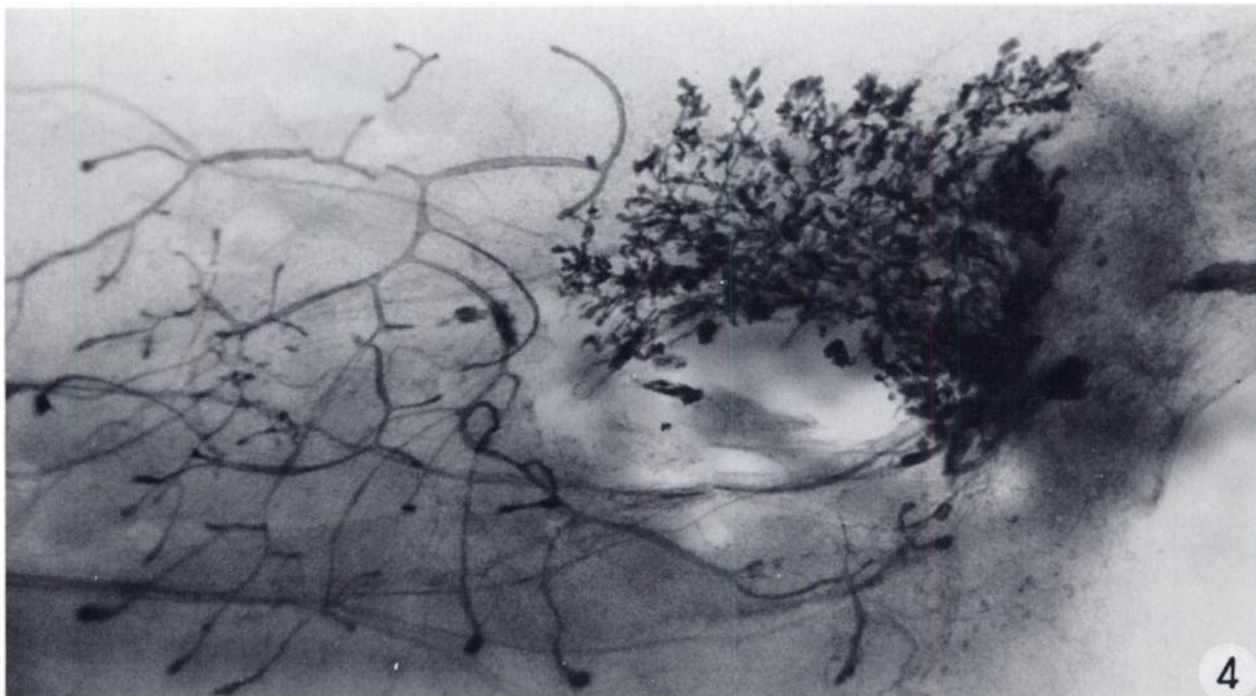
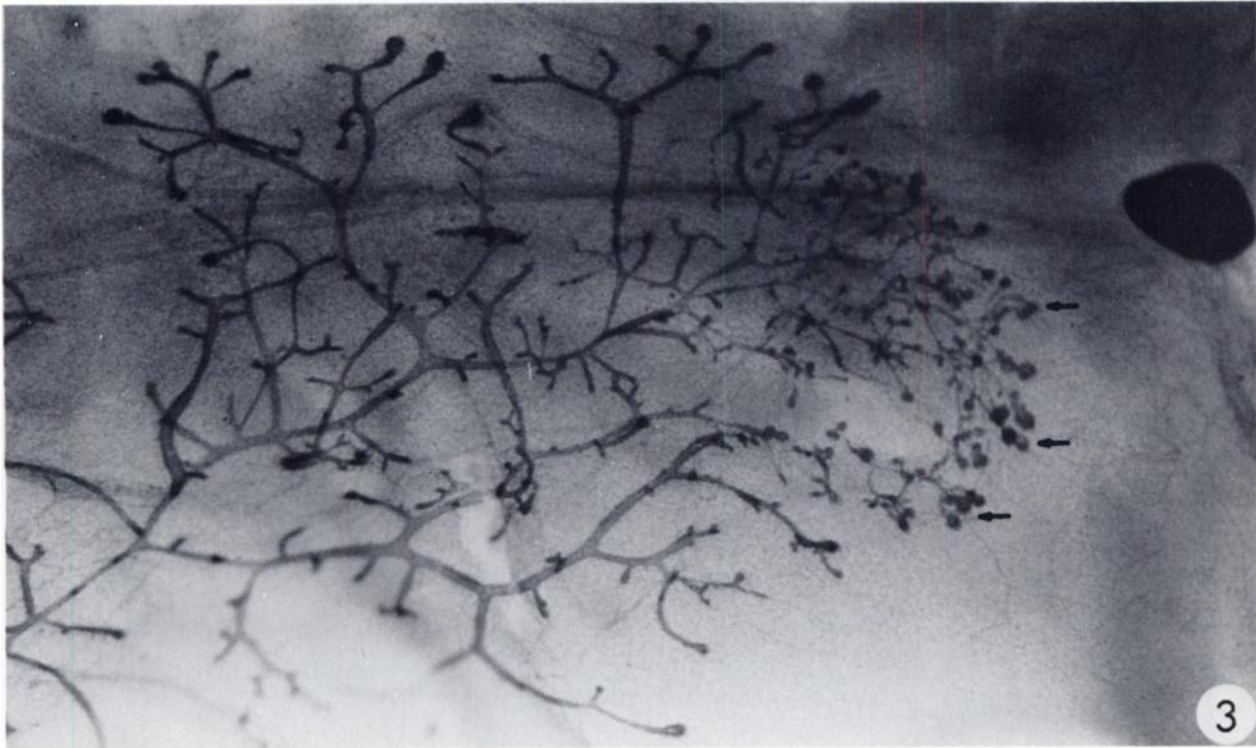


Fig. 3. Whole mount of a Class II lesion derived from 10^4 cells of a donor exposed to 0.075 mg of DMBA. Several hyperplastic end buds are visible on abnormal terminal structures (arrows).

Fig. 4. Whole mount of a Class III lesion derived from 10^4 cells of a donor exposed to 0.075 mg of DMBA. Alum carmine, $\times 20$.

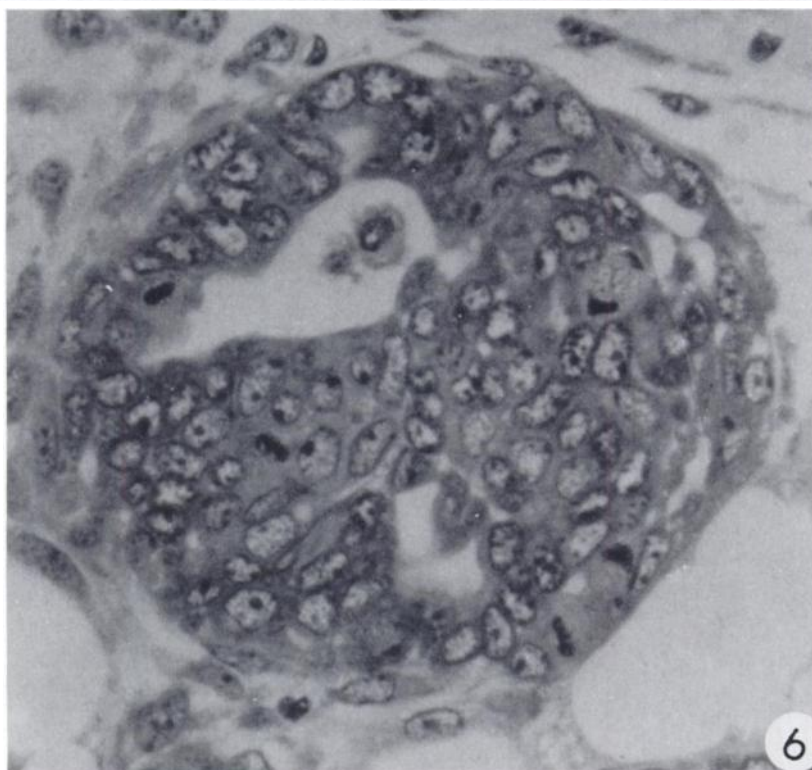
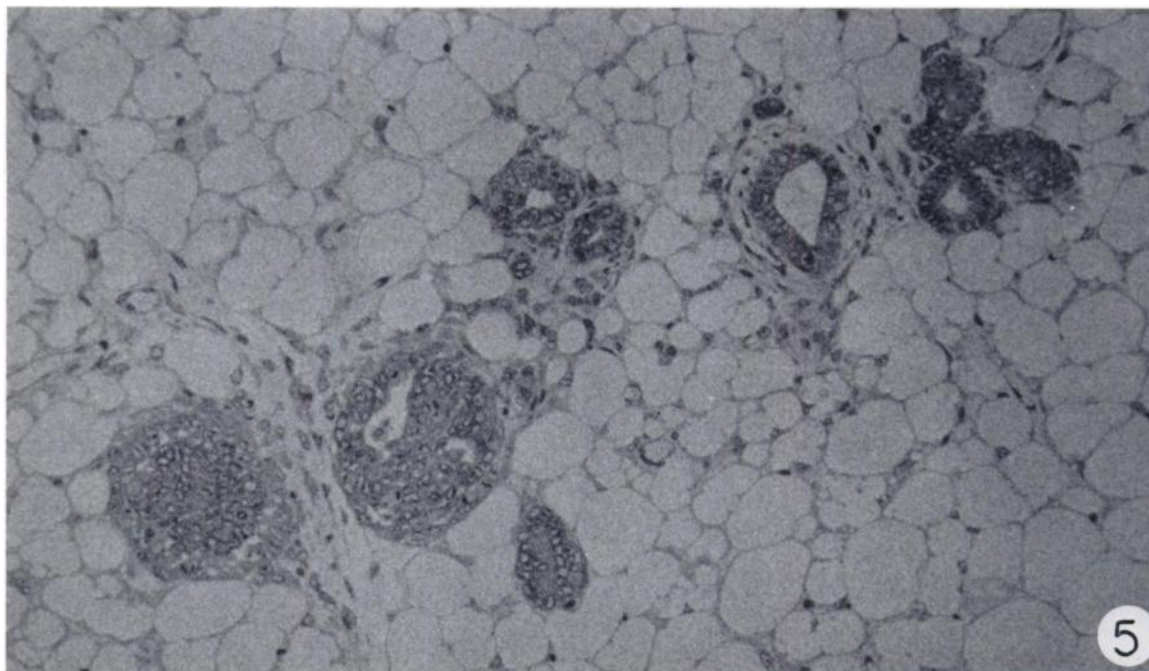


Fig. 5. Histological appearance of a ductal lesion. Methylene blue and basic fuchsin, $\times 100$.

Fig. 6. Higher magnification of ductal lesion shown in Fig. 5. Note the infolding of epithelial cells, cellular pleiomorphism, and numerous mitotic figures. Methylene blue and basic fuchsin, $\times 400$.