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Neoplastic transformation of epithelial cells in whole mammary gland *in vitro*

(organ culture/7,12-dimethylbenz[*a*]anthracene/carcinogenesis/mammary cells/BALB/c mice)

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ABSTRACT **Biological characteristics of nodule-like alveolar lesions (NLAL) induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in organ culture of whole mammary gland (BALB/c female mice) were assessed after transplantation into gland-free mammary fat pads of syngeneic virgin mice. (*i*) Tissue-fragment explants from NLAL areas of the gland produced abnormal lobuloalveolar (LA) outgrowths in 3 of 10 fat pads. (*ii*) Transplantation of dissociated cells of NLAL-derived LA outgrowths into 36 fat pads showed 100% LA outgrowths and 3 (8%) of these 36 outgrowths produced mammary carcinomas. (*iii*) The explants of dissociated cells from whole mammary glands treated with DMBA in culture produced full or partial LA structures in 2 of 56 outgrowths. (*iv*) The explants of dissociated cells prepared from outgrowths derived from explants as in *iii* produced 9 LA outgrowths in 16 instances; mammary tumor incidence in these outgrowths was 3 of 16 (18%). (*v*) The explants of tissue fragments from LA outgrowths as in *iv* produced LA outgrowths in 20 of 20 fat pads; mammary carcinomas appeared in 16 of 20 (80%) of these outgrowths. No NLAL was detectable in control glands treated with dimethyl sulfoxide (solvent for DMBA); explants of the control glands consistently produced ductal outgrowths and no tumor. This accomplishment of chemical carcinogen-induced neoplastic transformation of epithelial cells *in vitro* provides a model for studying carcinogenesis in an entire isolated organ.**

Use of the *in vitro* oncogenesis model of fibroblasts has produced impressive advances of knowledge about the pathogenesis of neoplasia (1, 2). Attempts to obtain transformation of epithelial cells in monolayer culture so far have been of limited success (3–5). However, induction of neoplastic transformation of cells in organ culture, in general, has remained a challenge, although 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced transformation in rat mammary slices in culture has been reported (6). Because human malignancies in different organs are mostly of epithelial cell origin, the need for an organ culture model for studying carcinogenesis *in vitro* has been emphasized in several reports (7–9).

By using the organ culture model of the whole mammary gland (10–14), it has been found (15–18) that treatment of the gland in culture with hydrocarbon carcinogens (DMBA or 3-methylcholanthrene) induces nodule-like alveolar lesions (NLAL). DMBA also binds to DNA of the mammary cells in culture, indicating that DMBA action in organ culture of the whole mammary gland is consistent with the characteristics associated with most hydrocarbon carcinogen-induced tumorigenesis *in vivo* and *in vitro* (2, 19). In this report we present biological characteristics, including tumorigenesis, of mammary epithelial cells treated with DMBA in organ culture. The findings provide a demonstration of neoplastic transformation of epithelial cells in culture of the whole mammary organ in a serum-free medium.

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MATERIALS AND METHODS

Animals, Organ Culture, DMBA Treatment, and NLAL Induction. Low-mammary-tumor strain BALB/c female mice free of mouse mammary tumor virus (MuMTV) activity were obtained through the National Cancer Institute. The procedures used for organ culture of the whole mammary gland, DMBA treatment, and NLAL induction were as described (16, 18, 20).

Enzymatic Dissociation of Mammary Epithelial Cells. For cell dissociation (CD), mammary tissue (minced) was first incubated (37°C) in a solution containing 1% collagenase and 1% hyaluronidase in Waymouth's medium, and this was followed by treatments with Pronase and DNase (21). This method of CD yields mostly epithelial cells with about 80–90% viability as monitored by the trypan blue exclusion test (21).

***In Vivo* Transplantation of NLAL and Dissociated Cells.** The biological characteristics of the mammary cells treated with DMBA or dimethyl sulfoxide (Me_2SO; solvent for DMBA) in organ culture were determined *in vivo* by transplantation into gland-free mammary fat pads of syngeneic virgin hosts according to the standard procedure (22). Fig. 1 outlines the experimental protocol.

Examination of Outgrowths and Tumors. The outgrowths produced by the explants in the gland-free mammary fat pads at different times after transplantation were initially assessed in anesthetized (Nembutal) animals injected intraperitoneally with 0.75 ml of 0.5% trypan blue solution in 0.9% saline 24 hr before microscopic examination. The trypan blue staining facilitates tentative detection of the outgrowth (21). As needed, small fragments (≈1 mm) of the outgrowth were transplanted into gland-free mammary fat pads of another batch of young hosts. The stained preparation of the fat pad was microscopically analyzed to confirm the morphological type of the outgrowth. Animals were palpated regularly for tumors; characteristics of the tumors were assessed by histopathology. Growth potential of tumor samples was tested by transplantation into intact mammary glands (inguinal) and into subcutaneous sites.

RESULTS

The morphology (Fig. 1) of DMBA-induced NLALs in organ culture of the whole mammary gland was as observed earlier (16, 18). The present experiments also showed that 70–80% of the glands contained NLAL, with an average of eight lesions per gland. No NLAL was observed in control glands treated with Me_2SO. A similar high incidence of DMBA-induced NLAL in organ culture of the whole mammary gland also has been recently observed by Tonelli *et al.* (24).

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; NLAL, nodule-like alveolar lesion; MuMTV, mouse mammary tumor virus; CD, cell dissociation; Me_2SO, dimethyl sulfoxide; Du, ductal; LADu, mixed lobuloalveolar plus ductal; LA, lobuloalveolar; LAT, lobuloalveolar with tumor; HAN, hyperplastic alveolar nodule.

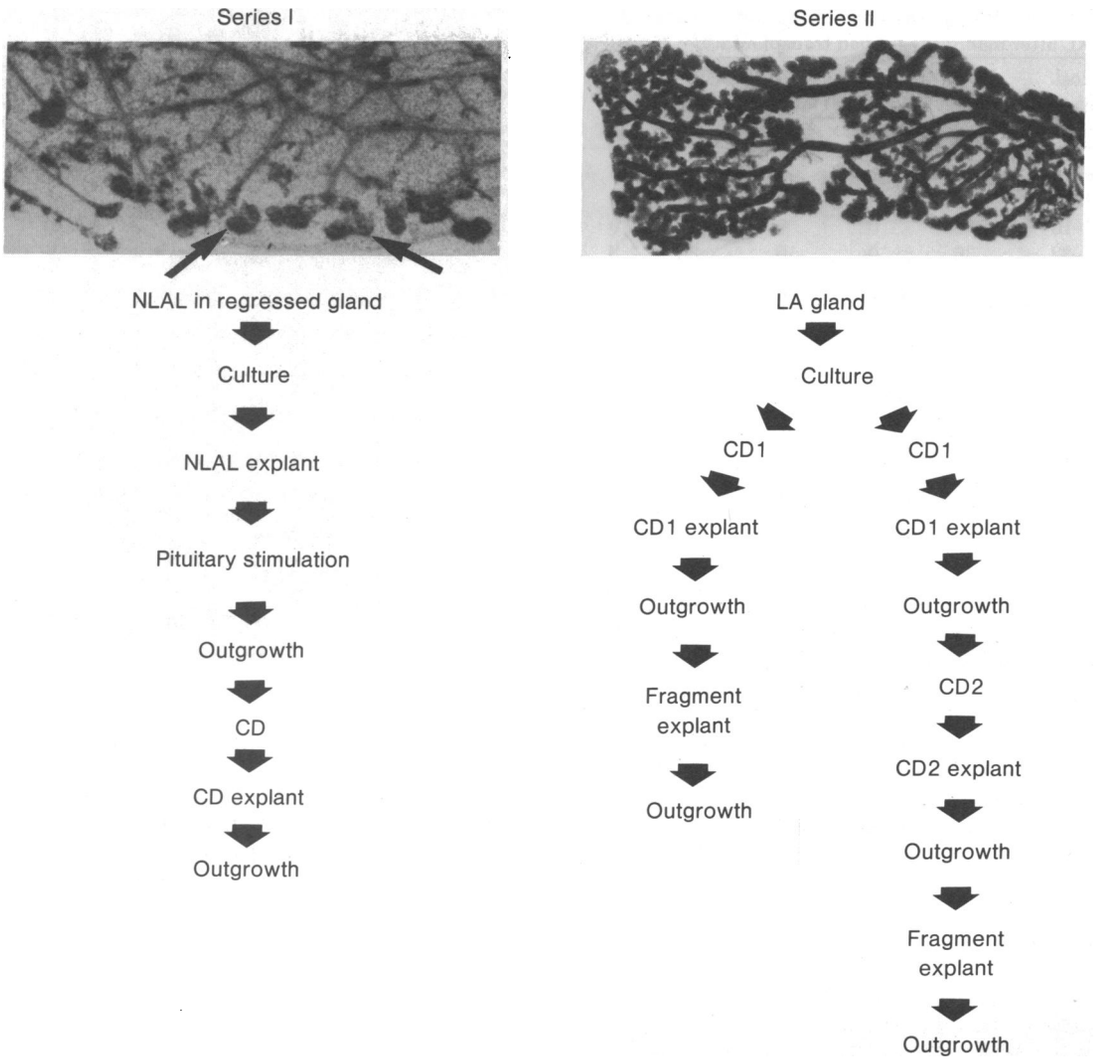

FIG. 1. Experimental protocol for assessment of the biological characteristics of the mammary cells treated with DMBA in culture of the whole mammary organ. (*Series I*) Whole second thoracic mammary glands of 3- to 4-week-old female BALB/c mice were cultivated for 9 days in Waymouth's medium containing insulin, prolactin, and hydrocortisone each at 5 μg/ml and aldosterone at 1 μg/ml. Between days 3 and 4 of culture the glands were treated for 24 hr with DMBA (7.8 μM); control glands were treated with Me_2SO (0.2% of medium). Subsequent 15-day cultivation in medium with insulin and aldosterone caused alveolar regression, and NLALs (indicated by arrow in the photograph) were present only in DMBA-treated glands (for details see refs. 16 and 20). One tissue fragment (fragment explant, $\approx$1 mm) from a NLAL area was transplanted into the right gland-free inguinal mammary fat pad of a syngeneic virgin host; the left side carried the control explant. Host animals bearing primary NLAL explants also carried a pituitary (from syngeneic male) isograft in the right kidney capsule during the initial 6 weeks after transplantation. [Pituitary stimulation during the initial weeks is conducive to expression of transformed cells in DMBA-induced precancerous hyperplastic alveolar nodules (HAN) of mouse mammary gland *in vivo* (23).] For preparation of CD explants, fat pads carrying NLAL-derived lobuloalveolar (LA) outgrowths (see Table 1) were pooled and subjected to CD. The CD explant containing 5×10^5 cells in 10 μl of Waymouth's medium was then injected into one gland-free inguinal mammary fat pad of syngeneic virgin host as described (21). (*Series II*) The initial 9-day cultivation and DMBA or Me_2SO treatments of the glands were as in series I. Whole LA glands (as illustrated) were subjected to CD. The dissociated cells contained in CD1 explants were then injected into the gland-free fat pads as in series I. For preparation of CD2 explants, the outgrowths from CD1 explants were subjected to CD. The CD2 explants containing these outgrowth cells were then injected into gland-free fat pads as in series I.

Assessment of NLAL Explants. Table 1 shows the characteristics of NLAL analyzed in series I experiments outlined in Fig. 1. After transplantation into gland-free mammary fat pads, fragment explants of NLAL areas produced ductal (Du), mixed lobuloalveolar plus ductal (LADu), and lobuloalveolar (LA) outgrowths in generation 1 (Fig. 2). Fragment explants of LA outgrowth obtained in generation 1 and carried through serial fat pad transplantation continued to produce LA outgrowths (generations 2 and 3A) in virgin hosts. Fragment explants of Me_2SO-treated tissue carried in contralateral fat pads of the host animals consistently produced only Du type outgrowth at transplant generations 1, 2, and 3A (Fig. 2*c*).

Mammary fat pad transplantation of dissociated cells of outgrowths derived from DMBA-induced hyperplastic alveolar nodule (HAN) of mouse mammary gland *in vivo* has been reported to elicit an enhanced expression of the latent neoplastic cells (25, 26). In the present study, CD explants prepared from NLAL-derived generation 2 LA outgrowths were transplanted into 36 gland-free mammary fat pads of virgin hosts. In generation 3B all 36 CD explants produced LA outgrowths, filling the fat pad within 8–10 weeks (Table 1). Mammary carcinomas appeared in three of these outgrowths between 2 and 5 months after transplantation (Fig. 3). CD explants prepared from generation 2 outgrowths produced by fragment explants from Me_2SO-treated glands produced only Du outgrowth in contralateral fat pads.

Table 1. Types of outgrowth derived from DMBA-induced NLAL after mammary fat pad transplantation

Generation	Host age, wk	No. of fat pads	Outgrowth type*			
			Du	LADu	LA	LAT
1†	8–48	10	5	2	3	0
2	8–48	9	0	0	9	0
3A	8–16	6	0	0	6	0
3B	8–32	36	0	0	33	3

* Du, ductal; LADu, mixed ductal and alveolar; LA, lobuloalveolar; LAT, lobuloalveolar with tumor.

† Pituitary stimulation during the initial 6 weeks and outgrowth were examined 10 wk after cauterization of the grafted pituitary. Generation 2 outgrowth was obtained from one of the generation 1 LA outgrowths (the remaining two were used for whole-mount preparation). Both 3A and 3B were generation 3 outgrowths, 3A from fragment explants and 3B from CD explants. Sixteen generation 2 LA outgrowths from a different batch of host animals were pooled for CD (for details see Fig. 1). Me_2SO-treated cells, both in fragment and CD explants, consistently produced only Du type outgrowth.

Assessment of CD1 Explants. Table 2 shows the results of CD1 experiments indicated in series II of Fig. 1. Among the total of 56 outgrowths derived from CD1 explants, 54 were Du, 1 was LADu, and 1 was LA in generation 1. Morphology of the single LA outgrowth was essentially as illustrated in Fig. 2*a*. Fragments from CD1-derived single LA outgrowth and from the LA region of the LADu outgrowth after retransplantation into gland-free fat pads produced similar LA outgrowth in generation 2 (Fig. 4 *a* and *b*). Fig. 4*c* illustrates a typical Du type outgrowth derived from CD1 explant from Me_2SO-treated control glands.

Assessment of CD2 Explants. As outlined also in series II of Fig. 1, CD2 explants used in these experiments were prepared from outgrowths produced by different batches of CD1 explants of DMBA-treated glands. Table 3 shows that, in experiment I, three of seven CD2 explants of 2×10^5 cells produced LA outgrowths and one of the LA outgrowths produced a rapidly growing mammary carcinoma (of the type shown in Fig. 3), 10 months after transplantation. In experiment II, six of nine CD2 explants of 1×10^5 cells produced dense LA outgrowths. Mammary carcinomas appeared in two of these outgrowths 6–8 months after transplantation. In control experiments, CD2 explants were prepared from outgrowths obtained from CD1 explants of Me_2SO-treated glands. The control CD2 explants in experiments I and II produced only Du outgrowths in contralateral fat pads. In experiment III, fragment explants from two of the LA outgrowths obtained in experiment II were retransplanted, one into the right and the other into the left gland-free fat pads of another batch of virgin hosts (Table 3). The dense LA structure in 20 of 20 outgrowths filled the fat pads in 8 weeks; within 4–5 months, mammary carcinomas appeared in 16 of these 20 outgrowths (Fig. 5).

Thus, 22 mammary tumors were observed in LA outgrowths produced by the explants of mammary cells treated with DMBA in organ culture. The tumors were transplantable into intact mammary gland as well as into subcutaneous sites.

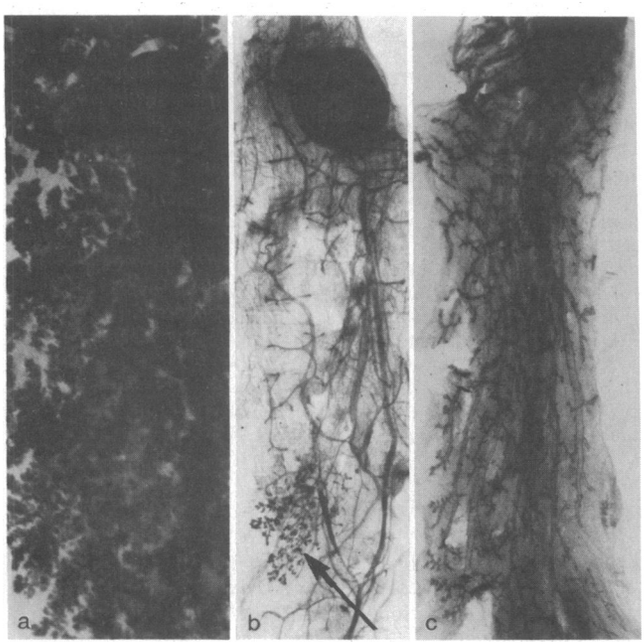

FIG. 2. Different types of outgrowths derived from NLAL explants. (×10.) (*a*) LA outgrowth of NLAL primary explant in virgin host 10 wk after withdrawal of pituitary stimulation. The characteristics are typical of the outgrowth obtained in subsequent transplant generations in virgin hosts. (*b*) Mixed LADu type outgrowth derived from a primary NLAL explant. The arrow points to the LA area. (*c*) Typical Du outgrowth derived from a primary explant of Me_2SO-treated gland in the contralateral fat pads.

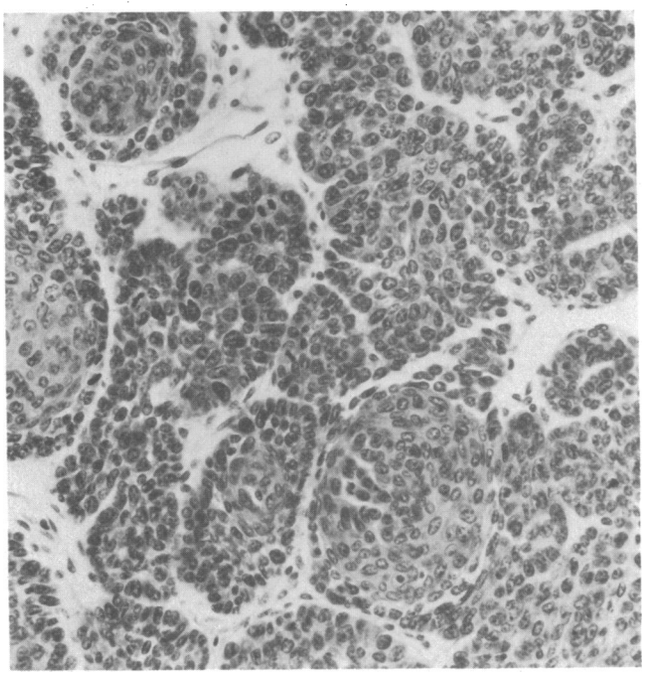

FIG. 3. Mammary tumor obtained from outgrowths of CD explant containing cells prepared from a NLAL-derived outgrowth line. (×200.)

Table 2. Types of outgrowth derived from CD1 explants of DMBA-treated glands

Exp.	Host age, wk	No. of fat pads	No. of cells in explant	Outgrowth type			
				Du	LADu	LA	LAT
I	8–48	20	1×10^5	19	1	0	0
II	8–48	12	2×10^5	11	0	1	0
III	8–48	24	5×10^5	24	0	0	0

An average of 75–85 DMBA- or Me_2SO-treated glands (36.8 mg per gland), pooled for each CD, yielded $\approx 4.9 \times 10^6$ cells. Me_2SO control explants produced only Du type outgrowths in contralateral fat pads (for details see Fig. 1).

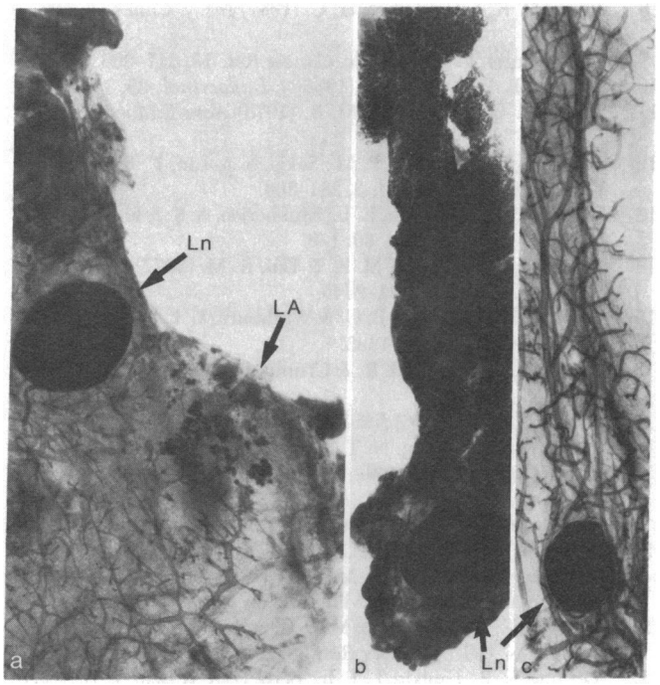

FIG. 4. Outgrowths obtained from CD1 explants. Ln, lymph node. (×10.) (*a*) LADu type mixed outgrowth. The arrow points to the LA area. (*b*) LA outgrowth derived from explants of LA area of the outgrowth in *a*. (*c*) Typical Du outgrowth derived from CD1, Me_2SO control explants.

DISCUSSION

The present findings on the ability of the NLAL explants to produce serially transplantable LA outgrowths in mammary fat pads of virgin host strongly indicate that the nodule transformation, associated with an escape of some mammary epithelial cells in NLAL from "normal" hormonal control, is irreversible and their characteristics appear to be similar to those of outgrowths derived from DMBA-induced HAN of mouse mammary gland *in vivo* (26, 27).

Furthermore, the appearance of several mammary tumors in the LA outgrowths obtained from NLAL-derived CD explants clearly demonstrates that neoplastic transformation of mammary epithelial cells is inducible by DMBA in organ culture of the whole mammary gland. The presence of latent neoplastic cells in NLAL further suggests that at least some of the NLAL induced in culture are potentially carcinogenic. Normal-appearing ductal outgrowths consistently produced by explants of Me_2SO-treated glands indicate that the transformation is a consequence of DMBA treatment of the mammary cells in organ culture. In an earlier attempt, intact

Table 3. Different types of outgrowths produced by CD2 explants after mammary fat pad transplantation

Exp.	Age of mice, wk	No. of fat pads	Type of explant	Outgrowth type Du	LADu	LA	LAT
I	8–48	7	CD2 (2×10^5)	4	0	2	1
II	8–48	9	CD2 (1×10^5)	3	0	4	2
III	8–32	20	Tissue fragment	0	0	4	16

The CD1 explants used in these experiments were prepared from batches of glands different from those in Table 2. In Exp. I and II, 16 and 12 outgrowths, respectively, produced by two different batches of CD1 explants were used. In Exp. III, tissue fragments from the two LA outgrowths obtained in Exp. II were transplanted into opposite gland-free fat pads. Me_2SO control explants in Exps. I and II produced only Du type outgrowth (for details see Fig. 1).

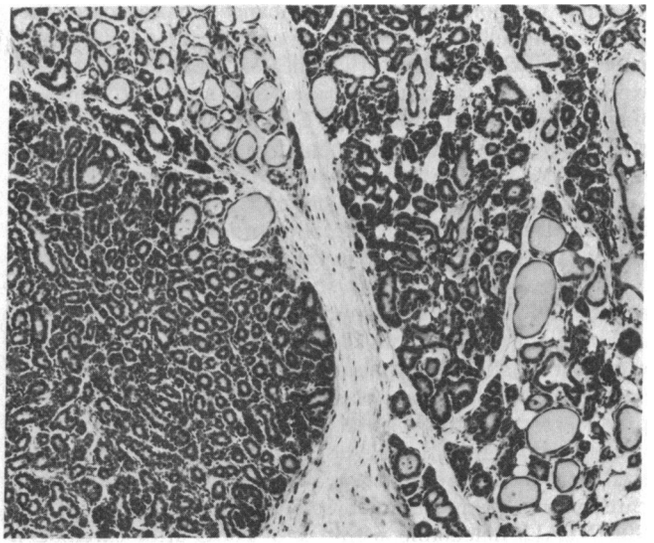

FIG. 5. Mammary adenocarcinoma in a typical hyperplastic LA outgrowth derived from CD2 explants. (×80.)

mammary gland (rat) exposed (20 min) to DMBA in culture was found to produce mammary tumor on transplantation *in vivo*, but carryover of the hydrocarbon into the site of transplantation remained a distinct possibility in this single preliminary experiment (28).

In CD1 experiments, only two explants of DMBA-treated glands showed abnormal LA or LADu structures. The LADu type outgrowths obtained from both NLAL and CD1 explants appear to resemble the LADu outgrowths derived from dissociated mammary cells of BALB/cfC3H (MuMTV+) female mice (21). Fragment explants of the LA outgrowth and LA region of the LADu outgrowth obtained from CD1 explants produced LA outgrowth in generation 2, indicating the irreversible nature of the transformation. The CD1 experiments, however, do not provide information as to whether the LA or LADu outgrowths are derived from NLAL or non-NLAL areas because the explants represent the total cell population of the glands pooled. Nonetheless, the morphological characteristics of the LA- or LADu-derived LA outgrowths appear to be similar to those derived from NLAL explants. Because CD1 explants of Me_2SO-treated glands consistently produced Du outgrowths, LA and LADu type transformations observed in the CD1 experiments again reflect responses of the mammary cells to DMBA action in organ culture.

Because the CD procedure is known to favor expression of inapparent transformed cells (21), the occurrence of LA and LADu characteristics in only 2 of 56 outgrowths derived from the CD1 explants appears rather low. However, recent findings (25) have indicated that mixing CD explants of HAN cells with dissociated normal mammary epithelial cells markedly decreases expression of transformed HAN cells, and the effect is directly related to the ratio of normal to HAN cells in the mixture. The CD1 explants in the present study are expected to contain mostly "normal" cells because in these experiments whole lobuloalveolar glands were used for CD (Fig. 1). Therefore, the presence of a vast excess of "normal" mammary cells may mask expression of the transformed cells in CD1 explants. Normal mammary cells are also known to have a finite life expectancy, whereas nodule-transformed (HAN) mammary cells have an unlimited proliferative potential (29). Thus, it is conceivable that the ratio of normal to transformed cells may be decreased during rapid spatial proliferation of the cells in CD1 explants filling the fat pad. Consequently, explants of CD2

cells may be more conducive to enhanced expression of the transformed cells. The appearance of typical LA outgrowths in 9 of 16 CD2 explants clearly indicates a markedly increased expression of the latent transformed cells, apparently present in outgrowths of CD1 explants. The expression of neoplastic cells is also favored in outgrowths derived from CD2 explants, as evidenced by the appearance of several mammary tumors. Furthermore, fragment explants of CD2-derived LA outgrowths after fat pad transplantation produced mammary tumors in 16 of 20 outgrowths in the following generation.

The results of the present studies demonstrate that both nodule and tumor transformation of epithelial cells are inducible by a carcinogenic chemical in culture of the whole mammary gland. The transformation is associated with the occurrence of discrete alveolar lesions (NLAL), some of which produce serially transplantable LA outgrowths having tumorigenic potential. In this respect, the NLAL appears to be analogous to the precancerous HAN induced by MuMTV or carcinogenic chemicals in mouse mammary gland *in vivo* (26, 30). NLALs detectable within 2–3 weeks after carcinogen treatment in culture thus may provide morphologic criteria useful for bioassay of potential carcinogens and drugs. NLALs are inducible also by carcinogenic aryl amines and amides (24), and a study indicates that retinoid inhibits DMBA-induction of these lesions (31).

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