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# Relationship Between Organization of Mammary Tumors and the Ability of Tumor Cells to Replicate Mammary Tumor Virus and to Recognize Growth-Inhibitory Contact Signals In Vitro

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Mammary tumor virus (MTV) replication was confined primarily to cells organized as acini in intact mouse mammary glands. Primary mammary tumors maintained a high degree of acinar organization and cells therein continued to replicate MTV vegetatively. Nonacinar mammary cells, derived by serial transplantation of acinar tumor cells, no longer actively replicated MTV. This suggests that phenotypic differences exist among mammary epithelial cells in their ability to support virus replication, that a fundamental relationship exists between the organization of epithelium for secretion and active virus replication, and that this relationship is not altered as a primary consequence of neoplastic transformation. Mammary epithelial cells from pregnant, non-tumor-bearing, MTV-infected BALB/cfC3H mice or from acinar mammary tumors from a number of mouse strains were grown in primary monolayer cultures. Such cell cultures under the influence of insulin and cortisol exhibited the ability to organize into discrete three-dimensional structures called "domes." MTV replication in such cultures took place primarily in cells within the organized domes. Cells cultured from nonacinar tumors did not exhibit any propensity to organize into domes, nor did they replicate MTV in primary culture. This suggests that the cell organizational requirement for MTV replication observed in vivo is conserved in primary culture. Dome formation is not an effect of virus replication, as cells from uninfected BALB/c animals organized into domes in culture without concomitant MTV replication. Growth-regulating signals, exerted between contiguous cells in cultures of non-MTV-infected mammary epithelium, were not modified by the occurrence of active virus replication nor as a direct consequence of neoplastic transformation. Cells derived from nontumor BALB/cfC3H glands and from spontaneous tumors exhibited cell growth kinetics, saturation densities, and deoxyribonucleic acid synthesis kinetics nearly identical to those of noninfected normal mammary epithelium in primary culture. Cell to cell growth regulatory signals were modified in cultures of nonalveolar tumor cells wherein evidence of overgrowth is documented.

Mammary glands of normal mice either contain acinar structures or contain cells which are potentially capable of giving rise to such structures under the influence of appropriate mammogenic hormones. The preneoplastic lesions occurring in mammary tissues of mammary tumor virus (MTV)-infected mice also are acinar, and most if not all mammary tumors develop from these nodules. Such tumors also maintain a high degree of acinar organization (5). However, it is well known that serial transplantation of acinar mammary tumors eventually results in the evolution of cells no longer able to organize into acini (10).

Electron microscopy studies of mammary tissues from MTV-infected mice have shown that budding of B particles, indicating MTV production, is confined essentially to cells which are

organized into acini (P. Nakayama, M. A. Thesis, Univ. of California, Berkeley, 1968). Recently, cells from spontaneous mammary tumors have been cultivated in monolayers. In response to insulin and cortisol, some of these cells organize into discrete three-dimensional units called domes (15, 16). Domes appear to be functionally analogous to acini in situ, since these are the only structures in vitro which are capable of abundant casein (*in preparation*) and MTV (15) production in the presence of appropriate hormones. Cells oriented in a two-dimensional monolayer are $10^{-3}$ to $10^{-4}$ times less efficient in the production of MTV.

These studies indicated that acinar organization might be a prerequisite to MTV "B" particle production in situ or in cell cultures. The present investigation was designed to determine whether the normal ability of mammary cells to organize into acini in situ was fundamental to their ability to organize into domes in culture as well as to their ability to produce MTV. The study was extended to evaluate the role of MTV replication in the conversion of normally growing cells to abnormally growing cells. For this purpose, contact (1) or density-dependent (22) inhibition of growth was used as a marker for normal cell growth control. The loss of this inhibition was considered to be an index of abnormal growth.

## MATERIALS AND METHODS

**Mammary tissues used for cell culture.** Primiparous, 5- to 6-month-old mice in early second pregnancy (less than 12 days) from MTV-free BALB/c ($C^-$) and MTV-infected BALB/cfC3H ($C^+$) strains served as donors of normal mammary tissues. The tissues from these mice are hereafter referred to as normal $C^-$ for normal BALB/c mammary glands and nontumor $C^+$ for nontumorous BALB/cfC3H glands. Electron microscopy studies have shown the presence of budding B particles in nontumor $C^+$ but not in normal $C^-$.

Primary tumors from three mouse strains were used: C3H, $C^+$, and RIII. Mice of these strains were all infected with MTV and tumors originated in multiparous females which were 8 to 12 months of age. In addition, tumors arising spontaneously in I mammary gland transplants in (C57 × I)$F_1$fC3H mice implanted with syngeneic pituitaries in their kidney capsules were also employed in several experiments. The origin of these I tumors has been described previously (20).

Spontaneous tumor cells from $C^+$ animals (spontaneous $C^+$ tumors) were transplanted after 7 days of growth in primary culture (for culture method, see below). Cells were scraped from culture vessels with a rubber policeman and inoculated into gland-free mammary fat pads of $C^-$ mice (4). These transplanted tumors will be referred to as transplanted $C^+$ tumors. Some of the I tumors referred to above were transplanted into I strain mice subcutaneously without intermediate in vitro culture. These tumors will be referred to as transplanted I tumors.

Pieces of tumors at each transplant generation were stained with hematoxylin and eosin for histological evaluation of epithelial cell organization. Pieces of tumors processed for electron microscopy were fixed in Karnovsky's (11) solution (1% formaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, *p*H 7.6) for 2 hr at room temperature. After storage overnight in buffer, they were postfixed in phosphate-buffered 2% osmium tetroxide, dehydrated in ethanol, washed in propylene oxide, and embedded in Epon 812 (14). The sections were stained with uranyl acetate and lead citrate (20) and were examined with a Siemens Elmiskop I or RCA EMU3F microscope.

**Cell culture technique.** The method (in preparation) for dissociation of normal epithelial (normal $C^-$ and nontumor $C^+$) cells is a modification of the collagenase method described by Lasfargues (12). After collagenase digestion of minced whole glands, the epithelium was partly separated from connective tissue cells by two differential centrifugations at 80 × *g* for 1 min in Eagle's minimal essential medium (MEM; reference 7) containing 2% fetal calf serum (FCS). Aggregated epithelial cells were further dissociated by trypsin (0.05%)-ethylenediaminetetraacetic acid (EDTA) (0.025%) treatment and rapid pipetting. Cells were then packed, resuspended in growth medium (*see below*), and plated at a density of $5 \times 10^5$ cells/cm$^2$. After this treatment, 80% or more of the cells in culture exhibited a polygonal epithelial morphology 2 days after plating.

The method of trypsin-EDTA dissociation and of plating mammary tumor cells has been described previously (16). Tumor cells were plated at a density of $5 \times 10^5$ cells/cm$^2$ in plastic (Falcon) containers.

Growth medium consisted of Eagle's MEM supplemented with 15% FCS, 10 μg of insulin per ml, and, in some cases where noted, 10 μg of cortisol per ml.

**MTV assay.** The method for assay of radioactivity in purified MTV has been described previously (15). Briefly, cells in primary culture were labeled with 10 μCi of $^3$H-uridine per ml (specific activity, 27 Ci/mmole; Schwartz BioResearch) for 24 hr. Labeled MTV was extracted and purified from culture fluids by a combination of discontinuous and isopycnic sucrose gradient centrifugation and then radioassayed. Total virus was calculated from the sum of radioactivity in the virus peak of 15 to 65% isopycnic sucrose gradients. Maximum radioactivity corresponded to a buoyant density of 1.17 g/cm$^3$ (±0.005).

This assay for cell-associated MTV antigens is an indirect immunofluorescence (IFA) test with rabbit antibody prepared against $C^+$ MTV whole virions isolated from milk. The method has been described earlier, and the specificity for antigens associated with MTV infection has been established (16).

**Cell kinetic and DNA synthesis measurements.** Cells were grown in plastic dishes (Falcon, 50-mm basal diameter) in Eagle's MEM plus 15% FCS and 10 μg of insulin per ml. At specified intervals after plating, cells were removed from culture vessels with trypsin (0.05%) in saline A ($Ca^{2+}$-$Mg^{2+}$-free BSS) and counted with a hemocytometer. Routine tests for cell viability

with trypan blue showed a frequency of dead cells of less than $10^{-6}$.

For deoxyribonucleic acid (DNA) synthesis kinetic studies, cells were grown on glass cover slips (15 mm in diameter) in 60-mm plastic petri dishes. Eagle's medium supplemented with 15% FCS and 10 $\mu$g of insulin per ml was used in cultures of these cells. Media were unchanged during the course of experiments. At prescribed intervals after plating, cells were labeled with $^3$H-thymidine (2 $\mu$Ci/ml; specific activity, 16 Ci/mmole, Schwartz BioResearch) for 1 hr. The medium was then decanted; cells were treated with 10% trichloroacetic acid for 1 hr at 4 C and then were washed three times (10 min/wash) with cold 10% trichloroacetic acid. Cultures were subsequently washed twice in 95% ethyl alcohol, air-dried, and radioassayed with a BBOT-2-methoxyethanol scintillation cocktail (21). Replicate samples for each time point were processed. Individual variation among samples within one time point was always less than 10%, even at the lower levels of radioactivity.

## RESULTS

**Classification of mammary tumors.** Tumors were classified (Table 1) as acinar or nonacinar by the criteria recommended by Dunn (6). Acinar tumors were those wherein a fine uniform acinar structure occupied over 50% of a tumor section. Squamous epithelium was of insignificant proportions. The tumors were composed predominantly of small cuboidal epithelial cells ordered in single or double rows surrounding cavities of various size (Fig. 1). Mitotic figures were rare. Spontaneous tumors of C3H, C$^+$, RIII, and I origin were almost always of this type (Table 1). Also, C$^+$ tumors retained the acinar character through seven transplants. Seventy-five per cent remained acinar at the eighth transplant. Nonacinar tumors developed after serial transplantation of acinar tumors and contained epithelial cells not arranged into acini. Cells were arranged as solid nests interposed among various amounts of connective tissue (Fig. 2). Mitotic figures were frequently observed. I tumors of the sixth to eighth transplant generation and 25% of BALB/cfC3H tumors at the eight and ninth transplant generation were of this type (Table 1).

**Evidence for MTV production in tumor cells in vivo.** The results of electron microscopy studies of all tumors are shown in Table 1. MTV B-type particles were found budding from epithelial cells organized into acini. MTV was always observed budding from microvillae at the luminal surface of these cells (Fig. 3). MTV B-type particles could not be found budding from cells of nonacinar tumors. Type A particles (2) were observed in samples of all tumor cells. Occasionally nonacinar cells in tumors classified as acinar showed some MTV budding into cytoplasmic vacuoles but not from the outer cell surface (eighth transplant acinar C$^+$ tumors).

**Evidence for dome formation and MTV production in primary cultures of cells from infected mammary glands and from acinar and nonacinar tumors.** Table 2 summarizes the dome-forming

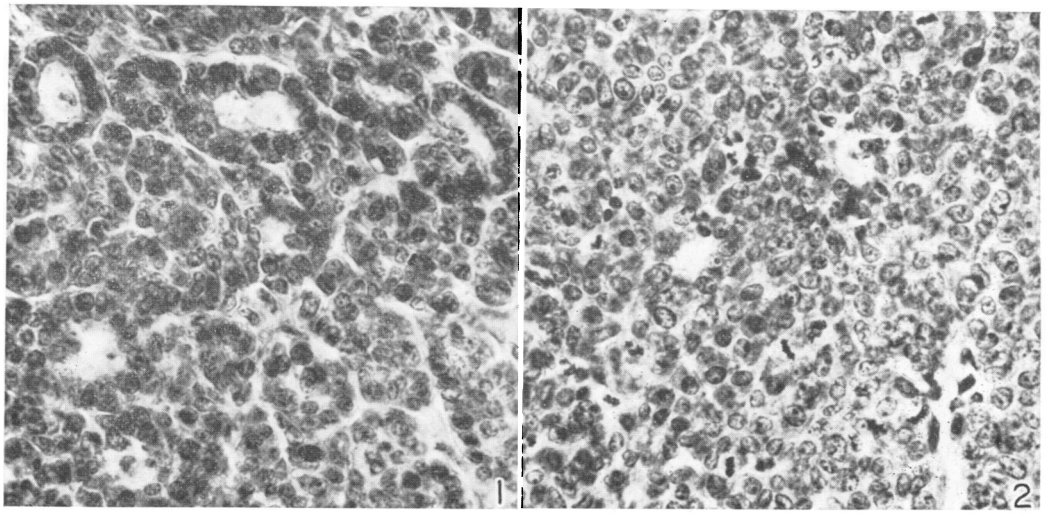

FIG. 1. *Section of a spontaneous tumor arising in a multiparous mouse of the BALB/cfC3H strain. Note that the tumor is predominantly acinar in organization and that only few mitotic figures are evident. Hematoxylin and eosin.* × *500.*

FIG. 2. *Section of a BALB/cfC3H tumor after the eighth transplant generation. Note that the tumor is nonacinar in organization and contains many mitocic figures. Hematoxylin and eosin.* × *500.*

TABLE 1. *Light and electron microscopic observations of mammary tumors*

| Strain of tumor origin | Transplant generation | No. of tumors | Light microscopic classification | Budding B particles[a] | Intracytoplasmic A particles[a] |
|---|---|---|---|---|---|
| BALB/cfC3H | Spontaneous | 20 | Acinar | Yes | Yes |
| BALB/cfC3H | 1–7 | 35 | Acinar | Yes | Yes |
| BALB/cfC3H | 8–9 | 12 | Acinar | Yes | Yes |
| BALB/cfC3H | 8–9 | 4 | Nonacinar | No | Yes |
| C3H | Spontaneous | 5 | Acinar | Yes | Yes |
| RIII | Spontaneous | 3 | Acinar | Yes | Yes |
| I | Spontaneous | 8 | Acinar | Yes | Yes |
| I | 6–8 | 7 | Nonacinar | No | Yes |

[a] Observations by electron microscopy; yes = 100% of sections through acini of acinar tumors showed B particles budding; no = no virus seen budding in a number of sections in excess of that number viewed for positive tumors. A particles were found in some samples of all tumors.

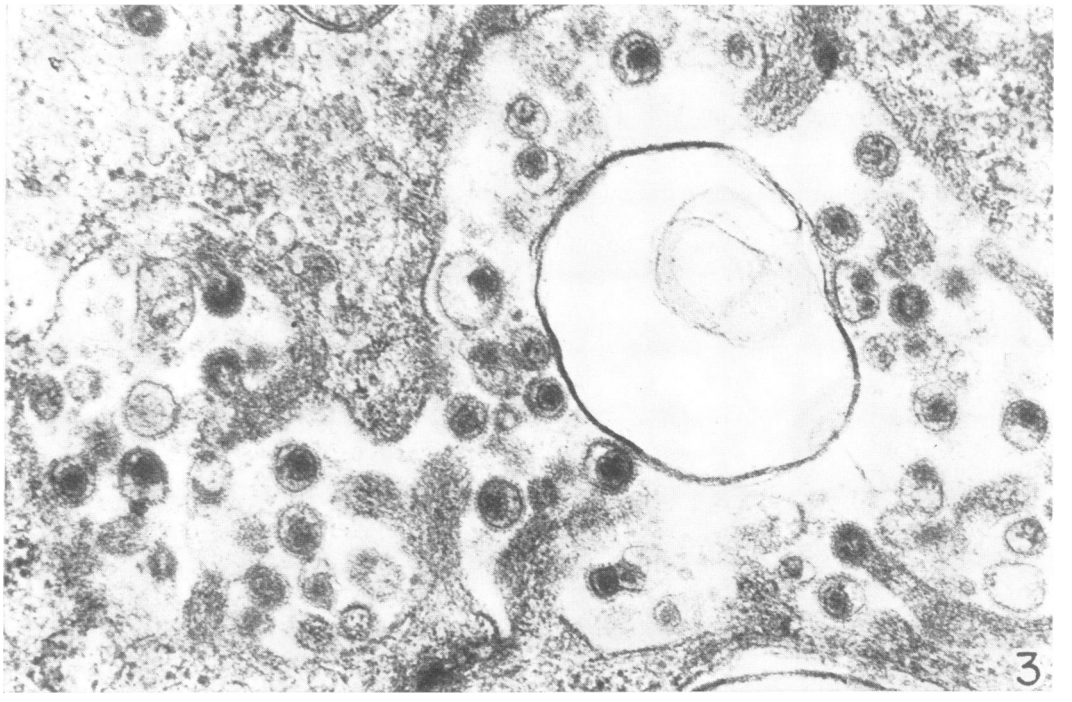


FIG. 3. *Electron micrograph of section from the tumor shown in Fig. 1. Note the presence of budding B particles at tips of microvilli and many mature B particles.* × *50,000.*

and virus-producing capabilities of cells derived from seven sources: spontaneous $C^+$ tumors, spontaneous C3H tumors, 9th transplant $C^+$ tumors, normal uninfected ($C^-$) mammary gland, nontumorous infected ($C^+$) mammary gland (all acinar tissue sources), 9th and 10th transplant $C^+$ tumors, and 6th to 8th transplant I tumors (both nonacinar tissue sources). Conditions for maximum dome formation described earlier (15) for spontaneous $C^+$ tumor cell cultures were used in these experiments: cells were plated at a density of $5 \times 10^5$ cells/cm$^2$ in Eagle's medium containing 15% FCS, 10 μg of insulin per ml, and 10 μg of cortisol per ml. These conditions were also optimal for dome formation in cultures of the other acinar tissues studied, and a maximum number of domes developed in cultures between 4 and 5 days postplating. Figure 4 illustrates the morphology of domes which developed in spontaneous $C^+$ tumor cultures.

TABLE 2. *MTV production and dome formation in primary cultures of normal and tumorous mammary cells*[a]

| Mouse strain | Tumor transplant generation | No. of tumors used | Histological classification | Average no. of domes/2.42 cm² at 4 days | Total virus yield (counts per min per $10^8$ cells) | MTV-positive antigens in domes/nondome cells |
|---|---|---|---|---|---|---|
| BALB/cfC3H | Spontaneous | 50 | Acinar | 480 | 6,600 | $443/10^{-4}$ |
| C3H | Spontaneous | 5 | Acinar | 364 | 5,400 | $301/10^{-4}$ |
| BALB/cfC3H | 9 | 6 | Acinar | 304 | 5,260 | $286/10^{-4}$ |
| BALB/cfC3H | 9–10 | 4 | Nonacinar | 0 | 108 | $0/10^{-5}$ |
| I | 6–8 | 8 | Nonacinar | 0 | 86 | $0/10^{-5}$ |
| BALB/c | Normal $C^-$ | | Acinar | 436 | 0 | 0/0 |
| BALB/cfC3H | Nontumor $C^+$ | | Acinar | 448 | 4,813 | $260/10^{-4}$ |

[a] Tissues from various sources were dissociated, and cells were plated in 75-cm² plastic flasks (Falcon) for $^3$H-uridine labeling and in 60-mm plastic dishes containing cover slips (2.42 cm² surface area) for dome counting and for indirect fluorescent-antibody staining. On day 4 postplating, domes were counted on replicate cover slips, and $^3$H-uridine (10 μCi/ml; specific activity, 27 Ci/mmole) was added to the flasks. Twenty-four hours after $^3$H-uridine labeling, medium was collected from flasks. Labeled MTV was purified from the fluids and radioassayed by the method described (15). Cells ($1.0 \times 10^8$ to $1.01 \times 10^8$) were labeled in all cases. Data are presented as total virus counts per minute present in virus peak in 15 to 65% sucrose gradients. Also, on day 4 postplating, 60-mm cultures were subjected to the indirect immunofluorescence assay for MTV antigens. The data are presented as the number of domes containing cells stained specifically for MTV antigens and the frequency with which such stained cells were observed in the nondome monolayer.

As shown in Table 2, cultures of all acinar tissues developed domes. Cultures derived from both nonacinar tissue sources (transplanted $C^+$ and transplanted I tumors) never exhibited any propensity for dome formation. Cultures of normal $C^-$ and nontumor $C^+$ cells exhibited as much potential for dome formation as did cultures derived from spontaneous $C^+$ tumor cells.

At the time of maximum dome formation for cells from acinar tissues, primary cultures of cells from all sources were assayed for MTV by the IFA and $^3$H-uridine incorporation techniques. A maximum amount of labeled virus was recovered from the fluids of cultures of spontaneous $C^+$ tumor cells after a 24-hr continuous label with $^3$H-uridine. No labeled virus was recovered from normal $C^-$ culture fluids, although the number of domes in these cultures was comparable to that found in spontaneous $C^+$ tumor cultures. Primary cultures of nonacinar tumor cells, which did not contain domes, released on 1 to 3% of the amount of labeled virus measured in fluids from cultures containing domes, i.e., cultures derived from acinar tissues whether tumor or nontumor. The number of cells labeled was nearly equivalent in all cases ($1.0 \times 10^8$ to $1.01 \times 10^8$ cells).

The results of IFA tests are shown in Table 2. As in the case of labeled cell-free MTV, ability to demonstrate MTV antigens was dependent on the organization of epithelial cells into domes. The percentage of domes containing cells stained for MTV antigens varied between 58% (nontumor $C^+$) and 92% (spontaneous $C^+$ tumors). The frequency of MTV-positive cells oriented in a monolayer in cultures containing domes was about $10^{-4}$ in all cultures of cells derived from acinar tissues. Cells derived from uninfected BALB/c mammary gland (normal $C^-$) showed no evidence of virus infection by this method. Primary cultures of cells derived from nonacinar tumors (transplanted $C^+$ and I tumors), which had lost the ability to form domes, also contained only a small number of cells staining specifically for MTV antigens (approximately $10^{-5}$).

**Kinetics of DNA synthesis and of cell growth in cultures of uninfected gland epithelium from $C^-$ mice, in infected normal epithelium, and in acinar and nonacinar tumors from $C^+$ animals.** Rates of DNA synthesis, measured as rates of $^3$H-thymidine incorporation into acid-precipitable material in cells during a 1-hr pulse, are shown in Fig. 6. Epithelial cells from infected nontumor $C^+$ glands and from acinar $C^+$ tumors incorporated thymidine at the same rate as normal uninfected ($C^-$) gland epithelium. The maximum rate of incorporation occurred at 30 hr postplating and declined sharply thereafter, so that by 60 hr the rate had returned to the low 6-hr level (4% of maximum at 30 hr). This level was maintained for an additional 36 hr.

Kinetics of DNA synthesis in cultures of cells from nonacinar tumors were similar to those described above until after the maximum rate was attained (30 hr postplating). The rate of decline in thymidine incorporation into DNA of

nonacinar tumor cells was less synchronous than in the other cell types and reached a lower limit approximately 25% of the maximum value (30-hr values). This level was achieved at 60 hr and was constant for an additional 36 hr.

The kinetics of cell growth (Fig. 7) in the three acinar tissue types were also quite similar. Doubling time was about 16 hr in all cases. Cultures were nearly confluent at 48 hr. The saturation density of these cultures was also the same, $8.00 \times 10^6$ to $8.01 \times 10^6$ cells per dish. These cultures remained stationary through 96 hr, a time during which MTV was actively replicated in cultures of infected cells.

Growth kinetics for nonacinar transplanted $C^+$ tumor cells were similar to those of acinar tissues up to 48 hr postplating. Although a saturation density of $8 \times 10^6$ cells per dish was achieved shortly thereafter for the acinar cell cultures, the nonacinar transplanted $C^+$ tumor cells continued to grow, but at a slower rate (about 50% of the 24- to 48-hr rate). By 96 hr postplating, the number of cells in the nonacinar transplanted $C^+$ tumor (Fig. 5) cultures was about double the number in cultures of spontaneous $C^+$ tumor and nontumor $C^+$ cells.

## DISCUSSION

The ability of acinar cells to organize into acinar structures, an activity fundamental to normal secretory activity, is maintained in MTV-infected nontumor mammary gland cells and in MTV-infected spontaneous mammary tumors. This ability is lost, subsequently, upon serial transplantation of acinar tumor cells. The ability of mammary epithelial cells to organize in vitro into domes was preserved in all cases in which in vivo acinar organization was manifest, whereas this ability was lost when the acinar organization of the parent tumor was lost. This suggests that the organization demonstrated in vitro is an expression of the natural propensity of mammary acinar cells to organize into three-dimensional hollow structures. The fact that trypsin-dissociated alveolar mammary tumor cells can undergo organotypic reorganization in vitro has been previously demonstrated by Dobrowska-Piaskowska (3). The observation that cells in domes produce casein after stimulation with mammotropic hormones (*in preparation*) provides functional evidence for domes being analogous to acini. Other characteristics of these structures have been described in more detail elsewhere (15, 16).

The fact that domes, indistinguishable from those occurring in nontumor $C^+$ and spontaneous $C^+$ tumor cultures, developed in cultures of non-MTV- infected BALB/c mammary gland suggests that the genesis of these structures is not under virus control. The data presented in this report, however, suggest that some parameter of acinar organization in vivo and dome organization in vitro is fundamental to the replication of MTV B-type particles. In vivo, MTV was shown budding from tumor cells arranged as acini but not from other cells, even when these were derived originally from acinar cells. In vitro, MTV, detectable either with antibody against whole virion antigens in an IFA test or by $^3$H-uridine incorporation into mature B-type particles, was produced primarily by dome cells in cultures of acinar normal (nontumor $C^+$) or tumor cells. About $10^{-4}$ cells not arranged in domes were active. Less than $10^{-5}$ cells in cultures of nonacinar transplanted I or $C^+$ tumor cells were active in this regard. Only low levels (1% of that found in dome cell cultures) of $^3$H-uridine-labeled virus were demonstrable in these cultures, although nearly identical numbers of cells were labeled in both types of cultures.

At the time of assay for MTV antigens or released virions, nonacinar tumor cells were growing and acinar tumor and nontumor cells were stationary. The inability to detect MTV in nonacinar tumor cells could then be due to an inability of MTV to replicate in dividing cells. It has been shown, however, in stationary cultures of acinar cells that nondividing nondome cells also do not replicate MTV to a greater extent than growing nonacinar, nondome cells (Table 2, Fig. 6 and 7; reference 17). This suggests that active MTV replication is not a direct function of the growing state of cells but rather a function of the organization of nondividing cells. Furthermore, it suggests that unregulated growth of nonacinar tumor cells is directly related to the inability of cells to organize into domes with consequent MTV replication.

Not all domes contained cells which reacted with anti-MTV antibody. Fifty-three per cent of the domes in nontumor $C^+$ cultures contained MTV-producing cells, whereas 90% of domes in spontaneous $C^+$ tumor cell cultures were active centers. The reason for the lack of 100% staining and the difference between these two cell cultures are not clear but may be related to the number of virus-infected alveolar cells in the two tissues. Additionally, it was not possible to assess the actual number of cells with MTV antigens in any individual dome. To accurately define the cellular distribution of MTV antigens in domes, these three-dimensional hollow shells of epithelial cells must be pressed to the plane of the monolayer. In such a two-dimensional orientation, it is usually impossible to delineate the original dome boundary. MTV-negative cells at the periphery

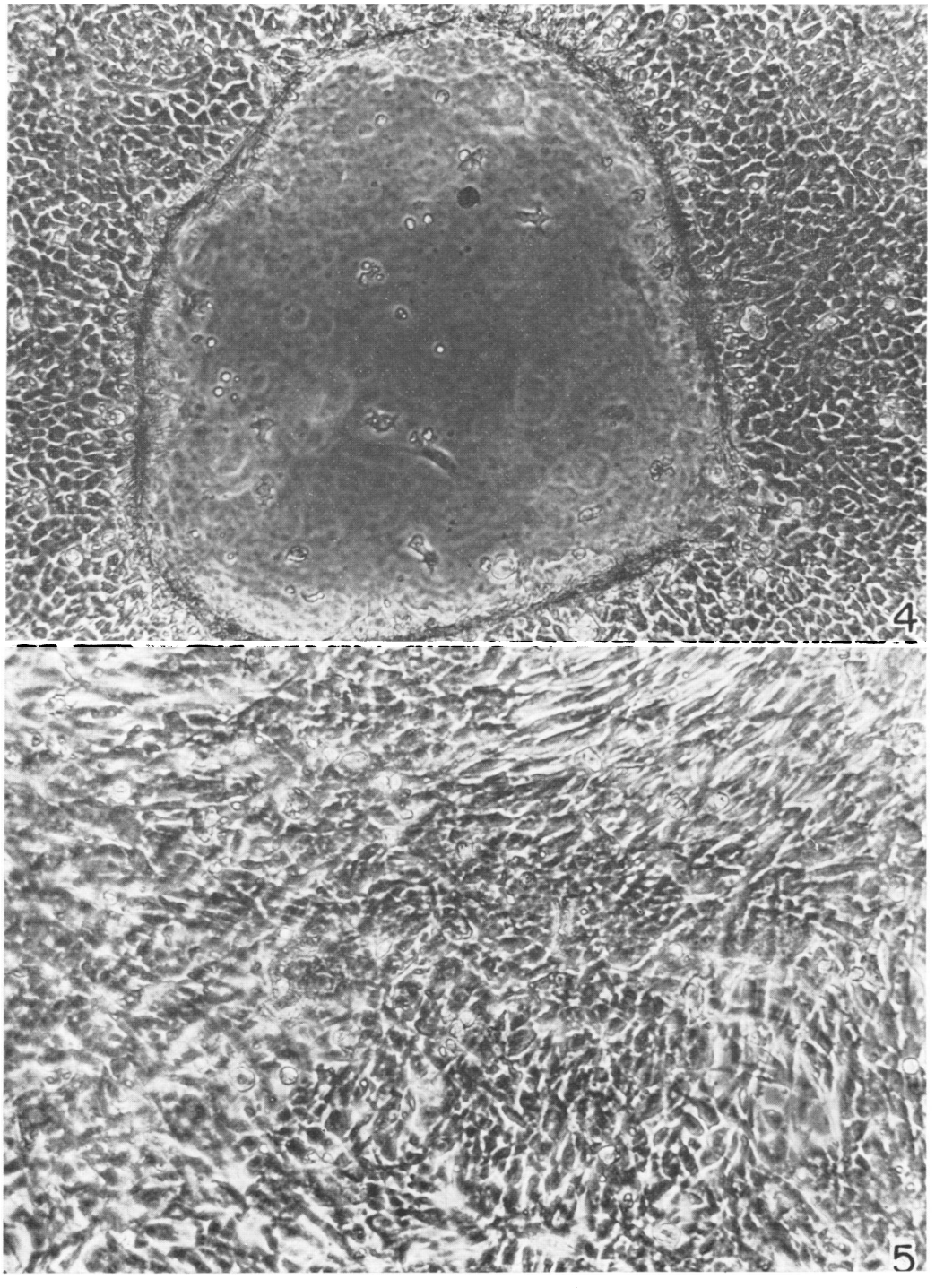

FIG. 4. *Portion of a 6-day primary culture of spontaneous BALB/cfC3H tumor. Note the presence of domes and the absence of cell overgrowth.* × *140.*

FIG. 5. *Portion of a 6-day primary culture of the ninth transplant nonacinar BALB/cfC3H tumor. Note the multilayered growth of cells.* × *400.*

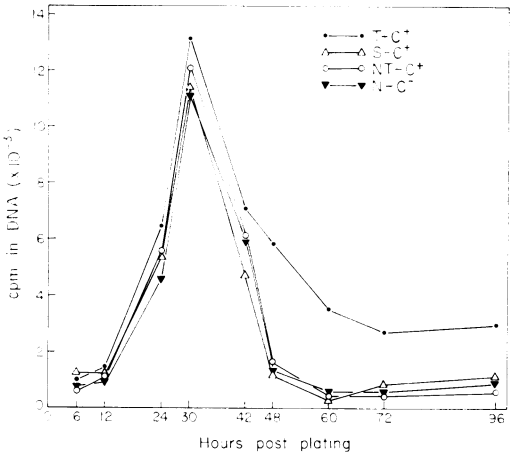


FIG. 6. *Rate of DNA synthesis measured as rates of $^3$H-thymidine incorporation into acid-insoluble DNA in nonacinar transplanted C+ (T-C+) and acinar spontaneous C+ (S-C+) tumor cells and in nontumorous C+ (NT-C+) and normal C- (N-C-) mammary tissues. Cells were dissociated from gland tissue or tumors. Dissociated cells (8 × 10$^6$) were plated in replicate 60-mm petri dishes containing cover slips 1.0 cm in diameter. Cells were grown in Eagle's medium plus 15% FCS plus 10 μg of insulin per ml. Cells were washed, and fresh medium was added at 6 hr postplating. At the intervals shown on the abscissa, 2 μCi of $^3$H-thymidine was added per dish for 1 hr. Labeled medium was then decanted. Ten per cent trichloroacetic acid was added for 2 hr at 4 C to precipitate DNA and to fix the cells. Replicate cover slips were removed from dishes, washed three times in fresh 10% trichloroacetic acid, washed two times with 95% ethyl alcohol, air-dried, and counted. $^3$H counts per minute at each point represent acid-insoluble thymidine in total cells attached to the cover slips. Each point represents the average of six replicates.*

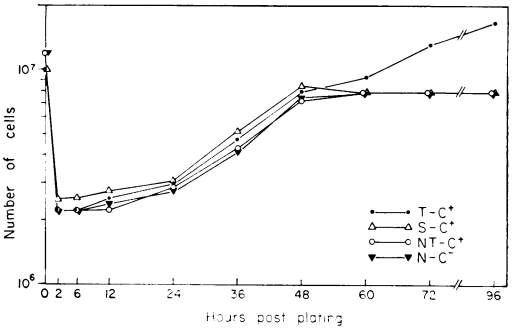


FIG. 7. *Kinetics of cell growth in nonacinar transplanted C+ (T-C+) and acinar spontaneous C+ tumor (S-C+) cells and in nontumorous C+ (NT-C+) and normal C- (N-C-) mammary tissues. Dissociated cells were plated in 60-mm plastic dishes at 10$^7$ cells per plate (in the case of tumors cells) and 1.2 × 10$^7$ cells (in the case of nontumor cells). Growth medium consisted of Eagle's MEM, 15% FCS, and 10 μg of insulin per ml. Cells were washed, and fresh medium was added at 6 hr. At various intervals over a 4-day period (abscissa), cells were removed from dishes with 0.05% trypsin and counted. Each point represents the number of viable cells per 60-mm dish at each interval postplating. Each point represents the average of three replicates.*

of an MTV-positive dome could be either dome or monolayer cells.

The insensitivity of electron microscope methodology in virus particle searches does not permit the conclusion that nonacinar cells are totally inactive in MTV production. The difference, however, between virus budding in acinar and nonacinar cells studied by this method is striking. The fact that sections of nonacinar tumors never exhibited extracellular B particle budding whereas the epithelium of nonacinar tumors was at least as homogeneous in nonacinar character as the epithelium of acinar tumors was in acinar character, wherein 100% of the sections exhibited extracellular B particle budding, strongly suggests that nonacinar cells may indeed be unable to replicate B particles. Moreover, the similarity in results between the electron microscopy studies and the more sensitive IFA and isotope methods in vitro suggests that the apparent absence of demonstrable MTV budding in nonacinar cells in situ does not simply result from insensitive methodology.

No judgment can be made as to whether nonacinar cells still carry MTV. No attempt was made to analyze these cells for subvirion components. Perhaps antibody with reactivity to internal MTV virion antigens would have detected MTV components. A-type particles, considered by some to represent progenitors of B-type particles (2), were found in samples from all tumor sources.

In contrast to the present findings, at least two established lines of cells have been described as producing MTV, one derived from a (C57 × Af)$F_1$ mammary tumor (23) and one derived from an RIII mammary tumor (E. Lesfargues et al., In Vitro **6**:376). In both cases, virus production does not appear to be influenced by the topographical organization of the cells, since MTV is replicated by cells oriented in a monolayer. If, however, in an intact, infected animal, the contrcls exerted upon MTV replication are linked with cell organizational control and virus is produced by cells physiologically organized for expression of differentiated mammary functions, then an in vitro system in which the same controls remain intact should provide

a more suitable system for the study of those controls.

If the ability of acinar cells to organize as such can be taken as an expression of normal acinar cell activity and the inability to organize as an abnormal activity, then this study has shown that MTV production is associated with this normal cell activity which is not lost in spontaneous tumors of the mouse strains tested.

MTV replication and release from cultured cells did not appear to modify the growth control of mammary cells in our experiments. Virus producing, nontumor $C^+$ and spontaneous $C^+$ tumor cells in cultures showed a high degree of contact inhibition comparable to that demonstrated by normal $C^-$ cells grown under similar conditions. Growth rates were nearly identical for all cultures during the log phase of growth, although the plating efficiency for normal $C^-$ and nontumor $C^+$ was about 5% lower than for tumor cells.

The fact that cultured cells from spontaneous acinar tumors did not grow to a significantly higher saturation density than normal gland epithelium seems paradoxical in view of the fact that cells in spontaneous tumors do overgrow in forming the tumor. However, it should be noted that in acinar tumors, the unit of overgrowth is the acinus itself; the tumor appears to grow by adding acini. Individual cells within developing acini grow and divide only until the acinus is formed. Very few mitotic figures can be identified in tumor acini once formed, and these structures do not grow to a size larger than normal acini. On the basis of histological studies, Nicholson (19) and Foulds (9, 10) came to the similar conclusion that the organoid characteristics of the parent tissue are maintained initially in spontaneous mammary tumors and that the smallest biologically significant unit of such a tumor is not a malignant cell but a differentiating acinar structure. Thus the formation of acini within tumors is not unlike the growth of acini in normal glands in that cell division ceases in each unit once the unit is formed. The difference between tumor and normal appears to be in the number of acini which develop. The breakdown in growth regulation in primary tumors may be an inability of acinus progenitors to recognize spacing regulations imposed on acinar growth which normally fix an upper limit to the number of acini which can occupy a given area (8). The modification of this particular regulatory function might be a primary consequence of virus infection. When acinar tumor cells have progressed to a nonacinar form, there appears to be no organizational control over growth of individual cells. In such cases, the growth regulatory signals normally exerted upon contiguous cells are no longer in effect, as manifest in the propensity for the cells to overgrow in culture.

Although several attempts have been made to infect mammary epithelium in culture with exogenous MTV, these attempts have been uniformly unsuccessful. In many such cases, the assumption has been made that MTV infection would result in modified growth behavior of infected cells (13) similar to those modifications induced by other oncornaviruses (17). The data presented in this paper suggest that one reason for the lack of success may be that MTV infection does not cause a significant loss of contact inhibition of growth between individual cells. Studies are in progress to determine the means by which acinar growth is normally regulated in an effort to understand how this regulation might be modified by MTV infection.

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