A PARTICULATE BODY ASSOCIATED WITH EPITHELIAL CELLS
CULTURED FROM MAMMARY CARCINOMAS OF MICE
OF A MILK-FACTOR STRAIN*

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PLATES 5 TO 7

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The occurrence of mammary tumors in mice has been shown to be influenced by a transmissible agent, by an inherited tendency to develop breast cancer, and by hormonal stimulation of the gland tissue. Since the initial report of the existence of an extrachromosomal factor (1) and the demonstration by Bittner of its presence in the milk (2), many investigators have sought a definition of its mode of action, its transmission, and its character. Their results have shown the agent to have many of the characteristics of a virus (3-5). In order to define the nature of the agent more precisely, attempts have been made to study it with the electron microscope. At the time of this writing, we are aware of only two previous accounts of such efforts. Graff et al. (6) have examined the ultracentrifugate of milk of high-cancer and low-cancer strains of mice. In a brief note they report that the high cancer strain milk contains a "heavy particle" which "has virus-like dimensions." Passey et al. (7) made water extracts of desiccated normal and malignant breast tissue from mice of high- and low-cancer strains. In micrographs of material from high-cancer strains they found a particulate component about 200 Å in diameter which, they report, was not present in extracts of tissue from low-cancer strains. Both of these observations, the latter more than the former, are subject to the criticisms that the agent may be greatly altered by the preparation procedures and may be easily confused in microscopy with particulate elements present in the cytoplasm of all cells. A study of the cells themselves would be less subject to these criticisms and might, moreover, give some information on the mode of reproduction of the agent and its relation to the tissue cells.

Earlier reports from this laboratory have disclosed the suitability of cultured cells for electron microscopy (8) and have demonstrated that the method can be used not only for the study of new cytological detail (9) but can be applied as well to the identification of viruses in or on cells (10) and to the study of the special cytological features of malignant cells (11). It seemed worth while to

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use similar techniques in an investigation of the cells from mammary tumors of
the mouse. The following is a report of the initial efforts.

Materials and Methods

The cells for study were grown from explants of both spontaneous and transplanted tumors. In all, four spontaneous and two transplanted tumors have been used. The former originated in female mice of the high-tumor strain C3H, a strain in which the milk factor is known to be operative; the latter were fifth generation transplants of a spontaneous tumor¹ that also; arose in a C3H mouse. All tumors were typical adenocarcinomas of the mammary gland that is to say they had the character of the growths known to be determined by the milk factor.

Cultures were prepared on formvar-coated slide inserts in roller flasks by methods described already (8). The explants were placed in shallow clots made up of equal parts of nutrient and chick plasma diluted 1:4 with Tyrode's solution. The nutrient was composed of 5 parts Tyrode's, 3 parts human cord serum, and 2 parts chick embryo extract. Each set of cultures was made with tumor tissue from a single animal, and successive sets were separated by intervals of 2 weeks or more. Different lots of cord serum, plasma, and embryo extract were used on each set. When, after a few days of culturing, small sheets of epithelial cells were obtained, the explants were removed, the remaining cells were washed briefly in a slow stream of Tyrode's (pH 7.4) and then placed in the vapor of osmium tetroxide for fixation. After periods over OsO₄, varying from 2 to 24 hours, the cells were mounted on screens and dried for electron microscope examination. All the micrographs were taken with an RCA (type E.M.U.) instrument².

Observations

The culture conditions of the experiments seemed adequate, since within 2
days of culturing most of the explants showed surrounding sheets of epithelial
cells. These continued to spread during the 3rd and 4th days, at the end of
which time they were usually fixed. Occasionally the cells of an epithelial
sheet were observed to cytolyze rather suddenly to be replaced later by a new
growth.

A typical epithelial sheet is shown in Fig. 1. It is apparent that many of
the cells are thinly spread and hence satisfactory for electron microscopy.
Only small portions of a cell can be micrographed in any one exposure, so most
of the illustrations include only a minute area approximating that outlined
(A) in Fig. 1.

The electron microscope examination of preparations of the epithelial sheets
had not gone far before it was observed that numbers of a small, apparently
spherical particle were associated with some of the cells (Fig. 4). The charac-
teristic density and morphology of these particles set them off from the normal
cytoplasmic components (Figs. 5 and 7). In some cells they lay scattered in

¹ Designated as Law 916.
² The microscope was generously loaned to the project by Dr. R. M. Taylor, Director of
the laboratories of the International Health Division of The Rockefeller Foundation.
small numbers (Figs. 4 and 5) while in others they literally packed the cell (Fig. 3).

Thus far in the material examined, the particles have been found associated only with epithelial cells or fragments of these cells. They can be seen in all parts of the cell and are not especially abundant nearer any particular component of it. Occasionally they appear to be within the endoplasmic strands and mitochondria (Figs. 4 and 5), and a few micrographs have shown them in the area of the nucleus (Fig. 3). It is difficult, however, to be certain whether they are actually within these structures or merely superimposed, the latter relationship seeming more probable in the case of the nucleus. Figs. 4 and 5 depict the more ordinary random association, and micrographs such as these show that the particles are generally situated in the ectoplasmic substance of the cytoplasm which, in these well extended cells, forms a thin layer between the membranes.

Small patches of cell membrane, sometimes with great numbers of particles on them, have been frequently found (Fig. 7). Evidently, while being washed prior to fixation, the cell proper was removed by the stream of Tyrode's and only portions of the membrane next to the formvar remained. The presence of the particles on such fragments indicates that in the intact cell they were located just within the cell membrane. Preparations of this sort are particularly valuable for good microscopy, since there is no overlying membrane and but little cytoplasm around the particles to scatter electrons and thereby reduce the definition. Similar fragments of cell membrane and other portions of the cell, probably products of natural cytolysis, have also been observed with associated particles, sometimes great numbers of them. Fig. 2 shows the particles definitely located on the cell surface. The significance of this observation is lessened, however, by the possibility that the cell may have ruptured in this region, releasing the particles shown.

The particles themselves are remarkably constant in character (Figs. 2 to 7). Their form appears to be spherical. Close examination of the micrographs (Figs. 4 and 6) reveals that most of them have a dark or dense center that is quite sharply defined and set off from a less dense, capsule-like periphery. This double structure is probably accentuated by OsO₄ fixation and may be consequent on the more osmiophilic properties of the central substance. In electron micrographs of gold-shadowed preparations it is possible in some cases (Fig. 5) to note that the central dense portion protrudes above a flattened border. This suggests that in desiccation the outer zone dries down more than the central part of the particle.

The diameter of the central, dense core is fairly constant from particle to particle and averages approximately 75 mμ (Text-fig. 1). The over-all dimension is more variable and averages about 135 mμ. Measurements on shadowed material indicate that the height of the dried particle is approximately one-half
VIRUS-LIKE BODIES IN MAMMARY CARCINOMA CELLS

the width. This is taken to mean that some flattening of the spherical form results from drying. The greater variation shown by the outside diameters (Text-fig. 1) is doubtless due in part to the fact that they are often poorly defined and not so accurately measurable. There is a suggestion in these findings as well that this portion of the particle is readily distorted, as if it were less rigid or viscous than the core.

The particles may occur singly, in pairs, or in clumps of all sizes. Some

![Histogram to show size distribution of particles.](image)

**Text-Fig. 1.** Histogram to show size distribution of particles. It was constructed from 238 measurements of the diameter of the inside dense portion of the particle and 303 measurements of the over-all diameter. The values of these two average approximately 75 μ and 130 μ respectively.

groupings are of special interest. In Fig. 6 (A), for example, one can see at least two rosette arrangements formed by a single row of granules surrounding a central, unusually large particle. Clusters of four bodies, two large and two small, as in Fig. 7, are fairly common. It should be mentioned also that some of the unusually large particles look as if compound in structure; i.e., composed of two, four, or more parts within a single capsule. The larger of such “encapsulated” clumps may have diameters almost double that of the average particle. Whether such special groupings of particles are stages in the reproduction of the typical form will have to be determined by further study. For studies of this type, the cultured material is especially favorable because
the particles are left relatively undisturbed and still associated with the tissue cell in which they are presumably multiplying.

Table I lists the origins of the material examined and shows the observed occurrence of the particles. It will be noted that in the preparations obtained from three out of six experiments none of the special sort described has been found. This does not prove that none was there, it means only that none was encountered during a moderately extensive examination of the screens. In the single preparation of transplanted tumor cells in which the particles were noted they were found only over a small area of the screen, in connection with a few cells. Screens from Experiment III (Table I), on the other hand, showed cells carrying great numbers of particles. Most of the illustrations were taken from this material. Particles were likewise more generally present

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Date</th>
<th>Source of tumor</th>
<th>Age of tumor</th>
<th>Mouse strain</th>
<th>Age of cultures when used</th>
<th>No. with particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Apr. 18</td>
<td>Law 916* Transplanted 5th generation 3 x 3 x 2 cm.</td>
<td>6</td>
<td>C3H‡</td>
<td>5-7</td>
<td>19</td>
</tr>
<tr>
<td>II</td>
<td>May 14</td>
<td>Law 916* Transplanted 5th generation 3 x 3 x 2.5 cm.</td>
<td>11</td>
<td>&quot;</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>III</td>
<td>Aug. 29</td>
<td>Spontaneous 1 x 1 x 1 cm.</td>
<td>2</td>
<td>C3H‡</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>IV</td>
<td>Nov. 24</td>
<td>Spontaneous 1 x 0.8 x 0.8 cm.</td>
<td>2</td>
<td>C3H‡</td>
<td>2-4</td>
<td>38</td>
</tr>
<tr>
<td>V</td>
<td>Dec. 4</td>
<td>Spontaneous 1.5 x 1 x 1 cm.</td>
<td>6</td>
<td>&quot;</td>
<td>3-8</td>
<td>14</td>
</tr>
<tr>
<td>VI</td>
<td>Dec. 12</td>
<td>Spontaneous 1 x 0.7 x 0.7 cm.</td>
<td>5</td>
<td>&quot;</td>
<td>3-6</td>
<td>40</td>
</tr>
</tbody>
</table>

*Law 916, transplantable adenocarcinoma that arose spontaneously in a high-tumor strain C3H mouse.
‡ Mammary tumor incidence approximately 90 per cent.
and abundant in the preparations of Experiment VI. Thus in these very limited studies the cells from spontaneous tumors derived from high tumor incidence C₃H mice have more commonly shown the granules than have those from transplanted tumors of similar derivation propagated in such mice.

**DISCUSSION**

These studies have disclosed the frequent presence in mouse mammary tumor cells of a particulate body, having a fairly uniform size, density, and morphology. Though similar in size, and possibly in other respects, to some normally occurring cytoplasmic granules (11), these particles are sufficiently different from the latter to give the impression of being special entities. Their uniform morphology, their association in closely packed clumps, as well as their irregular occurrence in tumor cells are especially significant as features distinguishing them from normal components of the cell. These same features make it seem probable that they are of extraneous origin and that they may be a virus.

The double structure shown by the particles cannot be explained as merely the image of a spherical body of uniform density. The latter, in an electron micrograph, would also show a relatively dark central portion, but its density should grade off gradually toward the periphery instead of suddenly altering to give a much lighter outer zone. Though evidence is lacking for any accurate interpretation of this finding, it does suggest the existence of a nuclear-like body surrounded by an envelope or capsule of different character. Electron micrographs of the viruses of equine encephalomyelitis (12), influenza A and B (13, 14), and vaccinia (15) have shown them to possess a similar complexity.

The variation in size recorded in the histogram (Text-fig. 1) is doubtless the reflection of a number of factors. Inaccuracy of measurement due to poor marginal definition will account for some of it; but the readily discernible differences in the diameter of the particles pictured make it clear that much of the variation is significant. As mentioned above, extremely large bodies have been seen that apparently consist of several small granules within a single "capsule." Possibly such large units are actually clusters of several small entities that have had their origin from a single particle. Conceivably, these small entities might enlarge to form separate particles of average size arranged around a primary one as in the rosettes shown in Fig. 6.

The most obvious, and for the moment, important question arising from these observations is whether or not the particles represent the milk agent of the mammary tumors. At present, there appears to be no simple and direct way of determining this; it will be necessary, instead, to gather evidence tediously through a comparison of tumor cells obtained from growths presumably carrying the milk agent with cells from the mammary tumors of agent-free animals. Such a study is in progress. Meanwhile, it may not be amiss
to consider the facts which favor identification of the particles with the milk agent. Numerous preparations of other cells from different species of animals, cultured in the same media as these mammary tumor cells, have not shown any particles of like appearance. It follows that they cannot have derived from the media. Secondly, in this connection, it seems significant that the particles are associated only with the epithelial cells of the cultures. Fibrocytes observed in the same preparations appear not to carry them. Other cell types from the mouse and from other species have been examined over the last 2 or 3 years, and no granules of precisely the same character have been encountered.

In view of these considerations and the fact that the cells were derived from tumors that arose in a high-tumor strain, it seems reasonable to assume tentatively that the particles are in fact the milk agent. In making this assumption we are aware of the possibility that the cells showing the particles may have come from tumors carrying an intercurrent virus. In this connection attention should be called to the tremendous difference in size between the particles reported upon here (1350 Å) and those observed by Passey et al. (7) in electron micrographs of material from mouse mammary tumors. In water extracts of tumors presumably carrying the agent these investigators found "approximately spherical particles about 200 Å in diameter" whereas in extracts of agent-free tumors nothing of the sort was observed. It is difficult to believe that this difference could be entirely a product of different preparation techniques. But further speculation may best be postponed until such time as Passey and his collaborators have tested the activity of their extracts and until the present studies of cultured cells have been extended to control material.

The electron microscope as a means for detecting intracellular inclusions is far from infallible, and unless the particles now under consideration are present in significant numbers or in clumps, they could be easily overlooked. This might account for their apparent absence in preparations made from some of the tumors, notably the two transplanted tumors studied. Yet it is possible that their scarcity in preparations from these latter represents a genuine scarcity. If so, the observation would tie in with some observations of Barnum, Ball, and Bittner (16) that the agent in transplanted tumors does not give as high a titre as in the spontaneous tumors.

It will be noted in Table I that in one set of preparations studied the particles appeared in the cells in tremendous numbers. The mouse from which these cells came was lactating at the time, and possibly this condition was attended by a multiplication of the agent. Conceivably also, the cells, if harvested an hour or two earlier, might have shown far fewer particles, whereas a little later they might have been destroyed.

Finally, it should be mentioned that a significant gain from these studies and our examination of normal cells, has been the experience needed for the
recognition of virus-like bodies associated with tissue cells. As one acquires the ability to discriminate the unusual or abnormal, the prospect develops that yet more pathological material can be examined with profit.

**SUMMARY**

Epithelial cells from spontaneous and transplanted mammary adenocarcinomas developing in high-tumor strain C3H mice have been grown *in vitro* and studied with the electron microscope. In preparations from three out of six tumors, an unusual particulate body has been found associated with the cells. The particles appear to have a spherical shape and a double structure consisting of a dense center and less dense outer zone. The diameter of the central dense portion is fairly uniform from particle to particle, averaging approximately 75 mµ; whereas the outside, whole particle diameter is more variable and averages about 130 mµ. From the micrographs it would appear that these peculiar virus-like bodies are situated chiefly in the ectoplasmic portion of the cell. They may occur singly, in pairs, or in clumps of varying sizes. Cells containing great numbers of the particles show signs of degeneration, and cell fragments are frequently encountered with many particles on them.

So far, the particles have been found only in association with the epithelial cells of the cultures. They are apparently not derived from the culture media. All in all the findings are consonant with the view that the particles represent the milk agent. Further evidence for or against this assumption is being sought from a study of cells from normal tissue and tumors demonstrated to be agent-free.

We are greatly indebted to Dr. Lloyd Law for his stimulating interest and the requisite material. It is a pleasure to acknowledge as well the technical assistance of Margaret Carr and Julia Hine.

**BIBLIOGRAPHY**

EXPLANATION OF PLATES

PLATE 5

FIG. 1. Photomicrograph of a typical sheet of epithelial cells grown from a spontaneous tumor explant. It illustrates the type of material that is suitable for electron microscopy. Small areas of approximately the size outlined (A) can be included in a single micrograph and the other figures are electron micrographs of such cell portions. The vacuoles are without significance in the present study. Taken from a 2-day-old culture, fixed in saline-formalin and stained with hematoxylin and eosin. × 200.

FIG. 2. Electron micrograph of a portion of a cell margin showing particles attached to the edge of the cell and to pseudopodia projecting from the cell. At the bottom of the micrograph is the cell body. It is possible that the particles on the outside have been released from the cell through disruption of the membrane. It is to be noted that outside as well as inside the cell the particles possess a double structure which may be taken to indicate that the “capsule” is an integral part of the particle. Preparation made from a 4-day-old culture of a spontaneous mammary tumor explant, fixed over vapor of OsO₄ 24 hours. × 14,000.

FIG. 3. Electron micrograph of a portion of a thick cell selected because it showed better than any other cell encountered in the preparation how gross the infection of particles may become. The large dark area outlined in white and shaped like a quadrant of a circular disc, which can be seen at the lower right-hand corner of the picture is part of the nucleus of the cell. The many particles which can be seen in this area may or may not be within the nucleus. Probably, as indicated by the definition of the image, they are between the cell and the nuclear membranes. Preparation from a 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO₄. Shadowed with gold at an angle of 10°. × 12,500.
(Porter and Thompson: Virus-like bodies in mammary carcinoma cells)
FIGS. 4 and 4 a. Electron micrograph of a small area of an epithelial tumor cell from a spontaneous C3H mammary gland carcinoma growing in culture. Fig. 4 a is a descriptive diagram of a portion of it. The particles occur singly or in clumps of various sizes and are scattered about without apparent association with any particular cytoplasmic component. The relatively large mitochondria lie amidst strands of the endoplasmic reticulum. The upper cell membrane seems to be intact. Apparently the particles are situated in the ectoplasmic substance between the cell membranes. Some of them can be seen to show a central density and a peripheral "capsule." Preparation from a 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO₄.  × 17,000.

FIG. 5. Electron micrograph of a cell similar to that shown in Fig. 4, demonstrating by means of gold shadowing the three-dimensional form of the dry particle contained in a cell. It can be seen that they have not dried down as much as the surrounding material and hence project up through the cell membrane, casting gold-free shadows. The mitochondria and strands of the endoplasm have flattened in drying. Some of the particles appear to be within the strands of endoplasm but may actually be superimposed. Preparation made from 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO₄. Shadowed with gold at an angle of 10°.  × 17,000.
**Plate 7**

**Fig. 6.** Electron micrograph showing several clumps of virus-like particles. These were found in a cell of a small epithelial sheet from an explant of a transplanted tumor. It can be noted that the individual particle has a dense center and a less-dense periphery, and that there is a distinct variation in size. Curious rosette arrangements of some of the particles are indicated by (A). Preparation from a 7-days old culture, fixed over vapor of OsO₄ 20 hours. × 21,500.

**Figs. 7 and 7 a.** Electron micrograph of a cell membrane with particles attached and descriptive diagram of a portion of it. During the preparation of the cells for fixation, the superficial part of the cell was washed away, leaving merely a small portion of the membrane next the formvar. One margin of this fragment runs across the upper left-hand corner of the picture, and its other edge across the lower right-hand corner. The finely granular background is the inside surface of the cell membrane. Presumably its granular character is due to structural units of the membrane or to adsorbed macromolecules. The line with the deep shadow to the right is the real edge of the cell and the full thickness of the latter can be seen extending upward. In this triangular lighter area both membranes are present. The virus-like particles are the larger white bodies. They stand up approximately spherical and cast long shadows. The numerous smaller and flatter biscuit-shaped bodies are vesicles of the cytoplasmic endoplasm such as are commonly seen in normal cells.

It can be noted that the shadowed particles show considerable variation in size. The indicated arrangement of two small and two large particles has been observed in other micrographs.

Preparation made from a 4-day-old culture, fixed 18 hours over vapor of OsO₄. Shadowed with gold at an angle of 10°. × 20,000.
(Porter and Thompson: Virus-like bodies in mammary carcinoma cells)