The Mode of Virus Elaboration in C3H Mouse Mammary Carcinoma as Observed by Electron Microscopy in Serial Thin Sections

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Summary

Thin serial sections were prepared for electron microscopy from the tissue of adenocarcinoma developed spontaneously in 8 C3H mice. They were submitted to 3-dimensional observation by means of graphic reconstruction.

It was found probable as a result that A particles formed as precursors of B particles in the cytoplasmic matrix of the tumor cell may either surround an individual vacuole or migrate to approach the surface of the tumor cell, and that they may then proceed to push up the unit membrane of the cell or the vacuole and may finally be pinched off into the glandular or vacuolar lumen to mature there into B particles.

The theory ascribing the process of virus formation to the budding of viruses direct from the surface of the host cell, which is based on an erroneous assumption, is not applicable in so far as explaining the mode of elaboration of viral particles found in C3H mouse mammary carcinoma is concerned.

Introduction

Bernhard et al. (6, 7) were the first to observe in an electron microscope that there were 2 different types of viral particles in mouse mammary carcinoma: the predominantly intracytoplasmic A, and the predominantly extracellular B, particles. Bernhard postulated that the former were formed in the cytoplasm as precursors of the latter and, moving later up to the cell membrane, were pinched off into the glandular lumen to mature there into B particles. This postulate has been accepted by Suzuki (33) who examined C3H mouse mammary carcinoma and by Pitelka et al. (31) who examined hyperplastic nodules produced in a C3H, C3Hf, and other strain mouse mammary gland. But it was rejected by Amano et al. (2, 4), Lasfargues et al. (22), and Moore et al. (26, 27), who held that the mouse mammary carcinoma virus made its first appearance as a bud direct from the cell membrane without any A particles migrating up to it. Some evidence apparently suggestive of this type of virus formation was obtained by Goldfeder et al. (12), who observed the same process in mouse mammary carcinoma, and by Miyawaki and Nishizuka (25), who noted it in hyperplastic nodules of the mammary gland.

Information seems to have been accumulating rapidly in recent years on the occurrence of a similar budding process in various kinds of neoplastic cells, such as the erythroblastosis cell (5), the Gross leukemia cell (11, 30), Moloney leukemia cell (10), Rauscher leukemia cell (32), the Swiss mouse leukemia and lymphoma cells (16-18), the Earle's L strain cell (8), the S mouse leukemia cell (3, 29), the Rous sarcoma cell (13, 19), and the virus-induced chicken nephroblastoma cell (20). More recently, however, Kinosita and Kakefuda (21) suggested that, in mammary carcinoma of the ICR strain mouse, the process of virus formation might be manifested first as an invagination of the unit membrane of the vesicle into the cytoplasm and later as a moving back of the particles into the vesicle lumen.

The argument about the mode of virus elaboration in tumor cells has thus not yet been brought to any acceptable conclusion, and the detailed sequence in which viral particles may bud has sometimes been differently postulated by different authors even when the tumor in question was mammary carcinoma. For instance, Amano et al. (2, 4), who examined mammary carcinoma in SL strain mice, noted that the budding began with the appearance of a peculiar filamentous structure beneath the cellular surface, whereas Lasfargues et al. (22) and Moore et al. (26, 27) reported that, in RIII strain mice, the protrusion and thickening of the cell membrane was the first sign of the virus formation. Amano and Ichikawa (2) pointed out further that the mode of virus formation might be different in different strains of mice, while Watanabe (34) confirmed that it was the same in all 3 strains he examined.

All the hypotheses cited above are based on the observations of the pictures presented in nonserial individual thin sections. No 3-dimensional scrutiny in serial thin sections has yet been performed by any investigator. We have recently succeeded in preparing the tissue of mouse mammary carcinoma into serial ultrathin sections. Examinations of these sections have disclosed that some of the pictures presented in nonserial sections have been grossly misinterpreted in the past. The results we have reached will be presented in the following pages.

Materials and Methods

The materials examined were tissues obtained from spontaneous mammary carcinoma produced in 8 C3H mice, 7 from the colonies kept at the Kyushu University, Fukuoka, and 1 from among those kept at the Tottori University, Yonago. Histologically, the tumors were each a moderately differentiated adenocarcinoma.

For electron microscopy, tissue blocks, not exceeding 1 mm in their greatest dimensions, were fixed for 1-1.5 hr. in 1% osmium tetroxide buffered at pH 7.6 with Veronal acetate and with

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Results

Answering to the description of Bernhard and others, the viral particles present in each tumor cell were of 2 types: the double-shelled A and the nucleoid-containing B particles, measuring 75—85 μm and 95—105 μm in diameter, respectively. The inner shell of an A particle was more electron dense than the outer. These particles A and B were identical in size and fine structure with the Type A2 particles described by Dalton (9).

The conventional description of the A particle as doughnut shaped was found erroneous on precise examination. In fact, a perfect doughnut-shaped ring was exhibited only in 24.4% of the A particles examined, and in 10.2% there was a break of continuity detectable somewhere in their double-shelled rings (Fig. 2). In the remaining 17.4%, the plane of the section in which the virus particles were cut was not adequate to show these particles in their true contours. The law of probability, when followed, in this case, on the assumption that each A particle has a pore 50 μm across its spherical shell, shows that each of some 13% of the A particles under observation has a pore visible in its electron microscopic picture, and that the above-mentioned figure—10.2%—is not significantly different from the corresponding figure—13%—statistically determined. The central core of each particle may be kept in communication with its outlying part by the pore. There was no evidence to prove the possibility that the horseshoe-shaped and the doughnut-shaped particle may be transformed one into the other.

The A particles were densely aggregated to form a viral cluster within the electron-dense matrix in the supranuclear cytoplasm, frequently in close association with cytoplasmic vacuoles of varying sizes. At the cellular surface, the A particles were found located frequently in microvilli and, rarely, beneath the cell membrane. There was present between the outer shell of each virus particle and the cell membrane an electron-dense zone of regular width—a zone approximately 10 μm in width. The B particles were found exclusively either in the lumen of the glandular structure or in the cytoplasmic vacuoles, many of them looking swollen and some containing 2 nucleoids each. The electron microscopic features of those particles which were disclosed when they were examined more closely in serial thin sections will be described in the following paragraph.

The nucleoid-containing B particle was the only type of viral particle found lying free in the glandular lumen. Some of them were in contact with the surfaces of the microvilli. Those particles which corresponded morphologically to the so-called immature B, namely the particles which looked apparently free or were barely attached to the cell surface by a thin pedicle, were confirmed by graphic reconstruction to be in direct substantial connection with 1 of the microvilli as shown in Figs. 4a—4d. They were nothing but A particles contained in the cytoplasm at the tip of each microvillus. These microvilli, some containing only 1 particle and others 2 or more, were each constricted regularly at the neck. No particles were identifiable as being intermediate morphologically between the free B and the intramicrovillous A particles.

Those A particles which were lying beneath the cell membrane were morphologically identical with those found in the microvilli or in the cytoplasmic matrix. Some of them had protruded so far into the glandular lumen that they pushed the cell membrane upward.

An electron-dense thickening of a cell wall or a vacular membrane which had been regarded by some investigators as an image presented by virus in its initial stage of formation was actually observed not infrequently in individual sections. But, when examined in serial sections, it was confirmed that this image was connected with a typical A particle (as described above) in the preceding or the following section, as shown in Figs. 5 and 6. It is obvious that the image in question was produced when an A particle was cut, not across its central, but across its peripheral part. Besides, relatively well-defined, double-shelled, crescent-like pieces were seen lying beneath the cell membrane or the vacular membrane (Figs. 8a—8d), which had also been regarded by some workers as precursory particles. Examinations in serial sections revealed, in this case, that the above-mentioned crescent-like piece was not an image produced by inadequate cutting of an A particle, but was a minor particle cut right across its center. Closer examination disclosed that the latter crescent-like piece was found located not only beneath the above-mentioned membrane, but also intermingled among the A particles clustered in the cytoplasmic matrix (Figs. 2, 7). The numerical ratio of these crescent-like pieces to all double-shelled particles was 10.7% at the vacular membrane, 7.6% at the cytoplasmic cluster, and 1.1% at the cell surface. More careful observation disclosed that among the A particles clustered in the cytoplasmic matrix were doughnut- or horseshoe-shaped particles disintegrating into twisted or broken fragments (Fig. 2). Some particles intermediate in shape and size between these fragments and the above-mentioned crescent-like pieces were also observed.

The relation between the intracytoplasmic A particles and the cytoplasmic vacuoles was noteworthy. The A particles associated with one vacuole were arranged in a row beneath the limiting membrane. Those associated with another vacuole in the same cell were so arranged as to push up the limiting membrane of the cell in different degrees and to force, as a result, the cytoplasm to protrude into the lumen of the vacuole; and those associated with still another vacuole were pushing up the membrane so markedly that the cytoplasmic projections containing the particles looked like so many microvilli. Some of the particles bore a close morphologic resemblance to the so-called immature B
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particles (Fig. 9). The pictures obtained from serial sections revealed that the topographic relation of each of these types of particles to the cytoplasmic projections was analogous to that observable between the same particles and the microvillus system at the cell surface (Figs. 9, 11, 12). A similar protrusion of A particles in the small vesicles, probably originating in the endoplasmic reticulum, was occasionally met with on a smaller scale. An inverse relation often existed between the number of A particles within the projections or of intravacuolar free B particles, on the one hand, and the number of A particles surrounding the vacuoles, on the other, indicating presumably that in the course of its elaboration the A particle may move periodically from the exterior to the interior part of the vacuole.

The thickening or the increase in electron density of the limiting membrane, or the presence beneath it of an ill-defined crescent-like piece, each an event of occasional occurrence, was interpreted as analogous in nature to the same finding at the cell surface.

Discussion

The mode of elaboration and release of viral particles in a tumor cell may vary according to the kind of tumor. The result of our examination of the C3H mouse mammary carcinoma appears to indicate that the A particles found in the cytoplasmic matrix of a host cell are identical morphologically with those particles in the microvilli of the surface of the cell or the vacuole, and that so-called “immature B” particles which appear to be lying free in a glandular or vacuolar lumen are A particles still confined to the tips of the microvilli.

It is generally agreed that the double-shelled (Type A) particles contained in the tips of the microvilli of a mouse mammary carcinoma cell may eventually be transformed into nucleoid-containing (Type B) particles in the glandular lumen (2, 4, 6, 7, 12, 27, 33, 34). This may lead us to the idea that the A particles in the cytoplasmic matrix may possibly be concerned in the process of B particle formation, because the former are quite identical morphologically with A particles present in the microvilli and because the same particles are occasionally found scattered between the matrix and the surface of the host cell.

The hypothesis of direct budding on which the mode of virus formation has been explained by Amano (2, 4), and by Moore and his co-workers (12, 22, 26, 27), is based primarily on the premise that one can trace in separate individual sections the successive stages of virus formation on the membrane of a host cell. However, the so-called “immature B” particles are recognized in serial thin sections to be nothing but the A particles contained in the cytoplasm at the tip of each microvillus. These findings may imply that the hypothesis of direct budding has lost one, if not all, of its major premises. Besides, those images which virus particles occasionally give when examined in nonserial sections, and which have often been believed to represent viruses in the initial stage of their formation are usually found, when examined in serial sections, to be images given by an A particle lying beneath the limiting membrane of the host cell and cut tangentially to its surface. It is an optical illusion to regard such an image as a virus in the making.

It is necessary to add some explanations on the double-shelled, crescent-shaped piece (Figs. 6–8). This piece may be taken at first sight as an A particle at an initial stage in its formation. However, it is not peculiar to the surface area of a tumor cell but is also found in the intracytoplasmic aggregate of A particles, as well as in the area surrounding a cytoplasmic vacuole of the host cell; it occurs most frequently in the former area (10.7%) and least often beneath the cellular surface (1.1%). These data may prove unfavorable to the theory of virus forma-
tion by direct budding at cellular surface. Presence of broken or twisted minor-sized fragments of A particles in their intracytoplasmic aggregate (Fig. 2) may imply that the double-shelled, crescent-shaped pieces are products of the disintegration of A particles, which may subsequently move to the vacuoles (in large number) or to the cellular surfaces (in small number).

The evidence accumulated in the course of our electron microscopic study using serial thin sections suggests that the elaboration of viral particles in C3H mouse mammary carcinoma may take place in the following sequence:

As suggested by Bernhard and others (6, 7), A particles may be synthesized in the cytoplasmic matrix of a tumor cell to form a viral cluster there (Chart 1, A). The A particles formed in the matrix, those lying near the cell surface in particular, may move up to the cell surface independently of the vacuolar system, some subsequently pushing the cell membrane upwards to make it protrude into the glandular lumen (Chart 1, B, C), others migrating into the preexisting microvilli (Chart 1, D), and all may eventually be pinched off into the lumen to be transformed there into mature B particles just as they are in the cytoplasmic vacuoles (Chart 1, F, G). Some of the vesicles located in the viral cluster may grow into vacuoles of varying sizes (Chart 1, b, c) and may subsequently push the A particles aside in all directions so arranged, in a row, they will closely surround the limiting membrane of each vacuole (Chart 1, d). The A particles may then begin to protrude into the vacuolar lumen and push the limiting membrane upward (Chart 1, e). Many microvillus-like projections which contain A particles may consequently be formed on the vacuolar wall (Chart 1, f). The A particles in these projections may eventually be pinched off into the lumen to be rapidly rearranged by an unknown mechanism into nucleoid-containing B particles (Chart 1, g).

The vacuoles so formed may move up to the cellular surface to open ultimately into the glandular lumen (Chart 1, H, h). The same process may take place on a small scale at each individual endoplasmic reticulum. The whole process may well be designated as maturation by migration. The migration of A particles has been referred to by a number of authors—Bernhard among the rest—and the possibility of occasional formation of B particles from A particles has not entirely been denied even by Moore, Goldfeder, and others.

The term “budding” may be applicable to this mode of migration as well, in that it describes not the direct budding of the cell membrane but the budding of cellular projections which contain migrating A particles. There is some analogy between the virosomes and the buds at the surface of the podocyte in avian virus nephroblastoma observed by Heine et al. (20).

The fact that there are few A particles between the cytoplasmic matrix and the cell membrane may possibly be due to the greater distance of the matrix from the cell membrane than from the vacuolar membrane. The fact may also be explained on the assumption that the rate at which the A particles move periodically towards the cellular surface may be relatively high.

The above-described minor-sized, double-shelled, crescent-shaped pieces (probably fragments of broken down A particles) are likely to migrate alike in the cytoplasm of the tumor cell. Those fragments formed within an intracytoplasmic aggregate of A particles are likely to move for the most part toward the membrane of the cytoplasmic vacuoles so that they become arranged in a row beneath it as the vacuoles grow larger and larger, while a few of the remainder migrate to the membrane of the cell. All of the crescent-shaped fragments probably disappear abnormally there, as there is no picture suggestive of a transition between them and any of the typical A and B particles.

Hairstone and co-workers (14, 15) have suggested that the smaller viral particle they saw in a strain A mouse mammary carcinoma might be identified as immature B particles. Matsui (23) and Nishiumi (28) have found similar particles in SL strain mouse mammary carcinoma, but they identified them as typical C particles. The present authors found similar particles of Type C in a spontaneous mammary carcinoma which occurred in a C3H mouse, but they have failed to recognize any particles transitional between them and the typical A or B. The immature B particles of Hairstone and co-workers may therefore be taken to represent some contaminated C particles.

To conclude, the result of our examination seems to support the classical view of Bernhard in so far as the elaboration of viral particles in C3H mouse mammary carcinoma is concerned, though there is at present no concrete ground for maintaining that the same explanation is applicable to all other varieties of tumor.

References


Fig. 1. Low power view of a glandular acinus in mammary carcinoma, showing the virus-cell interrelationship in the lumen of an acinus and those in the cytoplasm of the tumor cell. X 5500.

Fig. 2. An intracytoplasmic aggregate of A particles. Horseshoe-shaped particle (↓) and crescent-shaped pieces (↑) are seen scattered in the aggregate. X 60,000.
FIG. 3. A part of surface area of a cell. The letters a, b, c, and d denote the sequence of transformation from A particles to so-called immature B particles postulated by some authors. Close examination of serial thin sections reveals, however, that a particle with a constricted neck and looking free are each continuous with cytoplasmic microvilli. X 44,000.

FIG. 4. A part of surface area of a cell in 4 consecutive sections. The particles a, b, c, d, e, and f, which look liberated or about to be liberated in this picture, are found continuous with microvilli when examined in the serial thin sections. X 55,000.
Figs. 5, 6. Two sets of pictures taken from serial thin sections from different areas of the cellular surface. Images marked \( \downarrow \), looking crescent-like in the middle of 3 sections and lost to sight or left faintly visible as shadow-like figures in the remaining 2 sections, indicate that each is the picture of a real crescent-shaped piece. Images marked \( \uparrow \), looking crescent-like or like thickening of the cellular membrane in the middle of 3 sections and ring- or horseshoe-shaped in the remaining sections, indicate that those in the middle section are each cut in the peripheral part and not revealed in the complete original form. \( \times 14,000 \).
Figs. 7, 8. Pictures of 2 different serial thin sections, showing 3-dimensional shape of crescent-like pieces. Fig. 7. Cytoplasmic clusters of A particles in 4 consecutive thin sections; crescent-like pieces visible in the direction of the mark (\(\downarrow\)) in Section c and are not recognizable in any of the preceding or following sections. \(\times\) 24,000. Fig. 8. A particles on vacuolar membrane in 4 consecutive thin sections; a crescent-like piece is visible in the direction of the mark (\(\downarrow\)) in Section c and is not recognizable in any of the preceding or following sections. \(\times\) 37,000.
Fig. 9. Pictures indicating the relationship between cytoplasmic vacuoles and A and B particles. 

a: A particles aggregated in cytoplasm. X 19,000. 
b: Vacuoles increased in size among the aggregates of A particles. Many A particles are arranged regularly surrounding the vacuoles and a few have protruded into the vacuolar lumen; B particles are seen here and there inside the vacuoles. X 7,000. 
c: A particles protruding into the lumen of a large vacuole. X 7,000. 
d: A particles projected out of the vacuolar surface; microvillus-like projections containing A particles in its tip are also visible. X 45,000. 
e: B particles lying free inside a vacuole. X 17,000. 
f: Small vacuoles containing B particles exclusively, with cytoplasmic A particles aggregated at the upper left corner. X 25,000. (Scale = 1µ)
Fig. 10. Particles at the surface of a cell. Graphic reconstruction of serial thin sections. α, Five photographs are piled up; β, the same picture schematized diagramatically showing A particles contained within the cytoplasm. × 50,000.

Figs. 11, 12. Particles at the surface of a cytoplasmic vacuole. Graphic reconstruction of serial thin sections schematized to show the relation of A particles of different types to the vacuolar wall. × 50,000.

Fig. 13. A particles protruding into small vesicles probably originating in the endoplasmic reticulum to hold up its wall. × 95,000.
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