Ultrastructural and Quantitative Studies of Mammary Tumor Virus Production in Cultured Mouse Mammary Tumor Cells

Bernhard Kramarsky, Etienne Y. Lasfargues, and Dan H. Moore

Institute for Medical Research, Camden, New Jersey 08103

SUMMARY

A whole-cell-mounting technique was successfully applied to the study of mouse mammary tumor virus budding from cells in tissue culture. The technique permitted the visualization of ultrastructural detail, including the cores and the surface spikes of budding virions. The latter are difficult to discriminate in thin sections. Budding mammary tumor virus was compared with budding murine leukemia virus. The latter lacked the spikes and differed from mammary tumor virus with respect to the assembly of the cores. Binding of specific antibody to budding mammary tumor virions was directly visualized without the use of conjugated antibody. The antibody binding was found to be specific since the antibody was bound only to the virus bud and not to other regions of the cell membrane. Binding was not observed with control sera. The whole-cell-mounting technique was also used as an assay for virus production in infected cells. Synthesis of virus in tissue culture cells as a function of time was studied.

INTRODUCTION

The morphology of the mouse MTV 2 is especially well suited for study by the negative staining technique of Brenner and Horne (1). This method reveals ultrastructural details, such as the characteristic surface spikes which cannot be consistently visualized in thin sections. Choppin (2) studied the budding of influenza virus by negative staining of whole-cell mounts. Budding of virions from the cell surface is a distinctive feature of MTV synthesis; this aspect of MTV replication has not been studied by negative staining. The experiments reported here were designed to investigate the budding process with negatively stained whole-cell mounts of MTV-infected cells and to distinguish the budding mammary tumor virions from other virions, such as leukemia, which also reproduce by budding. Further characterization of budding mammary tumor virions and their differentiation from leukemia virus by direct electron microscopic observation of specific antibody binding (immuno-electron microscopy) were evaluated. The whole-cell-mounting technique was used as an assay for virus production in infected cells. Synthesis of virus in tissue culture cells as a function of time was studied.

MATERIALS AND METHODS

Cells

The initiation of the MMT cell line and of the MMT-derived lines MMT/C57, MMT/A, and MMT/R-5, as well as the methods of culture, have been described in detail elsewhere (3). MMT. This cell line was developed by Dr. John A. Sykes (8) from a spontaneous mammary tumor which arose in an F1 hybrid (C57BL X A) mouse. It has been in continuous tissue culture for more than 200 passages and continues to produce infective MTV. B particles have been observed budding from these cells by electron microscopy. MMT/C57, MMT/A, and MMT/R-5. These cell lines were obtained by alternate passages of MMT cells into animals and tissue culture. MMT/C57 resulted from passages into C57BL mice, MMT/A from passages into A mice, and MMT/R-5 from passages into Amsterdam/IMR rats.

Preparation for Electron Microscopy

The cells were prepared for electron microscopy by a technique adapted from the procedure described by Dales (3). Cell monolayers were trypsinized in BSS containing 0.05% trypsin and 0.002% Versene at 37° for 5 min. The cells were then sedimented by low-speed centrifugation and resuspended in tissue culture medium to give a suspension of approximately 2 million cells in 3 ml medium. This suspension was held at room temperature for 1 hr. The cells were then again sedimented and were resuspended in 0.5 ml of a hypotonic solution containing 1 part isonic PBS and 4 parts distilled water. This suspension was allowed to stand for 5 to 10 min at 4°. The cells were fixed in suspension for 20 to 30
min at 4° by the addition of 0.5 ml 2% unbuffered OsO4 and were then resedimented. The pellet of cells was washed twice with the hypotonic buffer solution to remove excess osmic acid and then resuspended in the same solution. One drop of this suspension was placed on a Formvar-coated 300-mesh copper grid and allowed to stand in order to permit the cells to settle onto the Formvar film. After removal of excess fluid with filter paper, the cells were stained with 2% sodium phosphotungstate.

Preparation for Immunoelectron Microscopy

The cells, suspended in tissue culture medium, were incubated for 1 hr as above and then sedimented by low-speed centrifugation. The medium was removed and the cells were resuspended in 2 drops of medium. Two drops of MTV antiserum, group-specific mouse leukemia antiserum, or normal rabbit serum, each inactivated at 56° for 1 hr, were added to the cell suspension. After incubation at room temperature with occasional shaking for 30 min, the tube was chilled and the subsequent operations were carried out at 4°. The cells were washed twice with PBS, resuspended in the hypotonic buffer solution, and prepared for whole mounting as described above.

Preparation of Antisera

MTV Antiserum. The procedure used was essentially that of Nowinski et al. (6). White New Zealand rabbits of either sex, approximately 8 pounds in weight, were immunized with 4 doses density gradient-purified MTV given at monthly intervals. Each inoculation consisted of the virus derived from 1.2 ml milk. The first 3 doses were emulsified with an equal volume of complete Freund’s adjuvant and injected into the thigh muscles of the rabbit (0.5 ml/thigh). The 4th and last dose was given without adjuvant. The rabbits were bled out 3 days after the final dose. This antiserum was absorbed with 2 volumes of intact C57 mice.

Leukemia Antiserum. Group-specific antimurine leukemia serum was obtained from Robert Nowinski of the Sloan-Kettering Institute, New York, N. Y. The preparation of this antiserum has been described (4).

Control Serum. Normal rabbit serum was prepared by bleeding white New Zealand rabbits.

Electron Microscopy

Negatively stained whole-cell mount preparations were observed in a Siemens Elmiskop I at 80 kV. Cells were viewed on the intermediate screen with a defocused condenser to select the cells to be studied. Only cells in contact with a grid wire were chosen since cells lying free on

The C57 skim milk was tested for the absence of leukemia virus by microimmunodiffusion, with the use of the leukemia antiserum obtained from Robert Nowinski.

Quantitative Determination of Virus Production

Cells were examined by whole-cell-mount electron microscopy for the presence of virus and were simply scored as positive or negative. A single virus bud sufficed for counting a cell positive. Cells were only counted as negative if one-third or more of their circumference could be observed. At least 120 cells were scored in each sample. “Productivity” of a cell culture, as referred to in this report, is defined as the ratio of cells with budding virus to the total number of cells examined.

RESULTS

Morphology of the Budding Virus. A large percentage of cells showed margins with numerous microvilli. Cells often showed virions budding at the tip or the lateral margin of a microvillus. Sessile budding at the cell margin was also common (Fig. 1). At higher magnification, the structure of these virions became evident. The spikes on the envelope were clearly seen. The core of the virion showed up as a light ring on a dense background. The center of the core often contained additional light regions (Figs. 2 and 3). Occasionally, virions could be identified on the surface of the cell (Fig. 4).

Under favorable conditions, the helical nucleoprotein of MTV was observed in virions which were in an early stage of budding (Fig. 5). The width of the helix was approximately 70 A, while the thickness of the coiled strand was about 24 A. These figures compare with the 76 and 27 A, respectively, reported by Sarkar and Moore (7) in helices obtained in preparations of Tween-ether-treated mammary tumor virus.

In order to compare these virions with C particles which are present in almost all, if not all, mice, we infected a culture of an established mouse embryo cell line with Rauscher leukemia virus and tested these cells by the same technique. Only a few leukemia virions were observed budding from the cell margins or from microvilli. These could be distinguished from mammary tumor virions since they lacked spikes and the cores usually showed an incomplete ring structure even when the virions were nearly separated from the cell. Free leukemia virions were frequently seen at or near the cell margin (Fig. 6).

Immunoelectron Microscopy. The binding of specific antibody by budding mammary tumor virions was observed by direct electron microscopic examination. Cells treated with anti-MTV serum showed electron-dense halos surrounding the budding virions. The precipitate was limited to the virus bud itself. Normal rabbit serum or antimurine leukemia serum failed to produce precipitate (Figs. 7 to 10).

Quantitative Determination of Virus Production. MMT/C57, MMT/A, and MMT/R-5 cells produce more virus than the MMT cells from which they were derived. Whereas MMT cell preparations rarely contained cells with more than 10
budding virions, the other 3 cell lines frequently contained many cells with 50 to 100 virus buds.

Initial experiments indicated that the productivity of MMT/C57 and MMT/A cells varied from culture to culture, while that of MMT/R-5 cultures was more consistent. The latter was, therefore, chosen for further study. A quantitative comparison was made of the productivity of MMT/R-5 cells with that of the MMT cells from which they were derived.

MMT and MMT/R-5 cells from fully grown culture were examined for the presence of virus by whole-cell mount electron microscopy. One million cells from each culture were passed into each of 8 T30 (Falcon Plastic Company, Oxnard, Calif.), tissue culture flasks. At 2- or 3-day intervals (Chart 1) cells from one of the flasks were examined in the same manner. The percentage of virus-producing cells varied with the age of the monolayer.

For the original MMT cells, the young sparsely grown cultures contained approximately 20% virus producers. After the cells were in tissue culture for 7 days, this percentage dropped to approximately 5%. After 11 days, the cells showed signs of degeneration and, by 14 days, only a few cells survived.

Cells of the MMT/R-5 line contained about 15% virus-producing cells after 2 days in culture. The percentage of producing cells increased to about 30% by 7 days and remained level until 11 days in culture. At about 14 days, there was a second increase in the percentage of virus producers to about 50%. The cells of this line did not degenerate with the 16-day period of the experiment and formed a heavy solid sheet. The results of this experiment are summarized in Chart 1.

**DISCUSSION**

Whole mounting of MTV-producing cells confirmed details of the fine structure of virus assembly and budding previously observed in thin sections of preparations of budding cells. Since in whole-cell mounts the entire virion is visualized, the structures observed can be interpreted without considering variations caused by the plane of section which may complicate interpretation of thin sections.

The fine structure of budding B particles was well preserved in preparations of negatively stained whole-cell mounts. Both the spikes and the internal structure were usually clearly visualized. The spikes were formed in an early stage of budding and the cores were usually completed even before the spikes were formed. While MTV virions in early stages of budding rarely had incomplete cores, in murine leukemia the core was usually incomplete until the buds had nearly pinched off.

Specific antibody binding by budding mammary tumor virions was visualized without the use of conjugated antibody. The specificity of the reaction was confirmed in two ways. (a) Control sera (normal rabbit serum and murine leukemia antiserum) failed to bind visibly to the virions. (b) The binding was limited to the area of the budding virus

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![Chart 1](chart1.png)

**Chart 1.** Production of MTV by MMT cells and MMT/R-5 cells.

\[ \text{Production} = \frac{\text{producing cells}}{\text{total cells}} \times 100 \]
MTV Production in Cultured Cells

The original MMT cells differed from MMT/R-5 cells with respect to virus production. The MMT cells produced significant amounts of virus only when the cell population was sparse, while MMT/R-5 cells produced best under conditions of crowding. The larger number and percentage of producing cells in the MMT/R-5 culture indicated that a much greater quantity of virus was synthesized by MMT/R-5 than by MMT.

The ability of the whole-cell-mounting technique to discriminate between the productivity of MMT and MMT/R-5 cells confirms the utility of the method as an assay for the ratio of producing cells in a culture. Fluorescent antibody staining, which has been used to measure ratios of virus-producing to nonproducing cells in infected cultures, may be subject to nonspecific effects and indicates the presence of viral antigens rather than complete virions in the cell. Whole-cell mounting directly and unambiguously determines the percentage of virion-producing cells in infected cultures.

REFERENCES


Figs. 1 to 10. Whole-cell-mount preparations, phosphotungstic stain.

Fig. 1. Low-magnification electron micrograph of the edge of an MMT/C57 cell. MTV particles (arrows) are budding at the tips of microvilli (MV). X 18,000.

Fig. 2. Higher magnification of an MMT/C57 cell. Virions (arrows) are budding at the tips and lateral margin of microvilli. X 90,000.

Fig. 3. A virion budding at the tip of a microvillus (MV) [MMT/C57 cell]. Viral envelope (VE), viral core (VC), and surface spikes (SP). X 120,000.

Fig. 4. Surface of a mammary tumor cell (MMT/R-5), Virions (arrows) are budding at the tip of short microvilli or directly at the cell surface. X 135,000.

Fig. 5. Two virions (arrows) in early stages of budding (MMT/R-5). The viral cores are incomplete. A nucleoprotein helix (enclosed) is seen in the core of 1 virion. X 425,000.

Fig. 6. Mouse embryo cell infected with Rauscher leukemia virus, Virions (arrows) are seen attached to or budding at the tip of a microvillus. The envelope is free of spikes and the core of the budding virion is not completely closed at double arrow. X 100,000.

Figs. 7 to 9. Mouse mammary tumor cells (MMT/R-5) treated with rabbit anti-MTV serum. Deposit of antibody on the virion (Ab). Virions are designated by arrows, X 120,000.

Fig. 10. Mouse mammary tumor cell (MMT/R-5) from the same cell sample not treated with antiserum. Virions (arrows) are not surrounded by deposits of antibody. X 120,000.
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