Stimulation of Mammary Tumor Virus Production in a Mouse Mammary Tumor Cell Line

Etienne Y. Lasfargues, Bernhard Kramarsky, N. H. Sarkar, J. C. Lasfargues, N. Pillsbury, and Dan H. Moore

SUMMARY

A mouse mammary tumor cell line (MMT), which has shown since its isolation in 1962 a limited, but continuous, budding of B particles, was stimulated to high production by alternate passages of the cells through mice or rats and tissue culture. Newborn C57BL, A, and A mice and Amsterdam/IMR rats have been used as transient hosts. Detection and semiquantitative estimations of virus production in the cultures were made by thin-section electron microscopy, whole-cell-mount electron microscopy, membrane immunofluorescence, and immunodiffusion.

Passage of the MMT cells into mice and back to tissue culture stimulated a 4-fold increase of B particle production but remained subject to variations in successive subcultures. Passage of the MMT cells into rats stimulated a 25- to 30-fold increase; budding of the virions remained constant and the tumor-inducing capabilities were high through multiple subcultures. The greater stability in pH and even rate of growth of the rat-passaged cells opens the possibility of producing in tissue culture a mammary tumor virus of greater reliability and purity than that obtained from milk or tumor extracts.

INTRODUCTION

One of the reasons for attempting to produce infective mouse MTV2 in tissue culture is to obtain a virus relatively free of the impurities normally associated with milk or tumor extracts currently used as source materials. The B particle, now well identified as the mammary tumor virion (9–13, 16), exists in both but is often associated with other viruses from which it is difficult to separate (8).

Attempts to obtain replication of B particles in cultures of mammary and embryonic tissues from normal mice have thus far been unsuccessful. By contrast, cultures of mouse mammary tumors produce an abundance of B particles early after explantation (7), but this production generally subsides after 1 to 6 months in vitro (15, 19). A MMT cell line obtained in 1962 by J. A. Sykes from a spontaneous tumor in a (C57BL X A) F1 hybrid mouse appears to be the only one to maintain a continuous production of B particles (18). During the 3 years in which we have maintained these cells, the production of B particles has been very low, although the fluid showed tumor-inducing activity when inoculated into assay mice. Recently, we found that alternate passages of the cells from tissue culture to mice and back to tissue culture increased the level of production of B particles. The present report describes the methods used and the results obtained in our efforts to produce larger amounts of MTV by tissue culture methods.

MATERIALS AND METHODS

Sykes' MMT cell line was obtained from the American Type Culture Collection, Rockville, Md., where it is registered as certified cell line 51 (CCL-51).

The culture medium recommended by Sykes consisted of Hanks'-Eagle's minimum essential medium enriched with 10% unactivated newborn calf serum to which insulin, penicillin, and neomycin in the respective amounts of 0.020 mg, 500 units, and 0.250 mg/ml were added.

Passages of the cells into mice less than 24 hr old were made by i.p. inoculation of 2 X 10⁶ cells in 0.1-ml aliquots. The cell suspension was inoculated with a tuberculin syringe and a 27.5-gauge needle into the peritoneal cavity through the left thigh to avoid leakage. A marked swelling of the abdomen, impairing movement, occurred within 1 week. The nodules which formed in the peritoneal cavity and the mediastinal region were excised and trypsinized in 0.25% trypsin and 2.5 X 10⁵ cells were explanted in plastic T30 flasks (Falcon Plastic Company, Oxnard, Calif.). The cultures were maintained through 3 or 4 serial transfers in the same medium as used for the original MMT cells and then reinoculated into newborn mice. To avoid confusion, the term “transfer” used in this paper applies to passage of cells in tissue culture, whereas the term “passage” designates cell passage through animals. Several in vitro-in vivo cycles were performed with C57BL and A mice, the 2 parental strains of the hybrid in which the MMT cells originated, and also with A mice. The latter were selected because they are genetically identical to A but carry a highly tumorigenic MTV.
Passage of the MMT cells in newborn rats were made under the same conditions except that the nodular growths resulted from s.c. inoculations. The Amsterdam/IMR rat used in these experiments was a pure inbred line obtained from the Gynecological Laboratory, University of Lund, Lund, Sweden, 6 years ago and tested by the skin-graft technique for genetic purity. Newborn rats have shown a temporary tolerance for Rous sarcoma cells, HeLa cells, and L-cells. MMT cells developed into large nodules within 7 days but regressed in 12 to 15 days.

Evaluation of MTV replication in cultures of mouse- or rat-passaged cells was made by electron microscopy, immunofluorescence, and immunodiffusion, 7 days after cell transfer.

Budding of B particles was observed by electron microscopy of thin sections of cultured cells fixed in 1% osmium tetroxide or glutaraldehyde and osmium tetroxide. The cells were embedded in Epon, sectioned on a Porter-Blum microtome, and observed in an A.E.I. Model EM6B electron microscope. Five blocks were prepared from each specimen and about 200 sections were examined from each block.

The whole-cell-mounting method described by Kramarsky et al. (6) was used for the semiquantitative appraisal of viral budding in freshly trypsinized cell suspensions. Since an average of 40 to 50 cells could be observed on a single grid, the approximate percentage of cells shedding B particles could be readily determined.

Monitoring of B particle production in all cultures was also done on negative-contrast dispersions of pellets formed after ultracentrifugation of 12 ml culture fluid at 32,000 rpm for 1 hr in a Spinco No. 40 rotor. From these frequent checks, although not quantitative, the changes in B particle production could easily and rapidly be followed. Quantitative particle counting was made from 100 ml culture supernatant. After a preliminary low-speed centrifugation, the culture fluids were spun at 21,000 rpm in an SW 25.1 rotor for 90 min. The pellets resuspended in 1 ml PBS were pooled and again centrifuged. The final pellet was resuspended in 0.2 ml PBS, mixed with 0.1 ml Latex particle suspension and 0.1 ml PTA containing 1% bovine serum albumin. The Latex particles of uniform diameter and known concentrations were used as a dispersion reference. After 5 min of low-frequency sonic oscillation, the mixture was sprayed on carbon-coated electron microscope grids.

Indirect membrane immunofluorescence performed on the same cell suspensions as used for the whole-cell-mount method also indicated the percentage of cells carrying MTV antigens. An MTV antiserum, prepared according to the specifications of Nowinski et al. (14), was applied to the cells as described by Klein et al. (5). It was also used in a modified microgel immunodiffusion test as described by Charney (3).

RESULTS

Morphology

The MMT cells are epithelioid but might appear in culture as elongated elements with slender cytoplasmic extensions forming a network structure. Freshly seeded cultures show, after 48 hr, groups of 5 to 10 cells forming small epithelial sheets interconnected by cytoplasmic bridges (Fig. 1). In older cultures, small spherical balls of tissue are frequently observed. Because of this pattern of growth, total confluence in a uniform monolayer is rarely achieved. The MMT cells present a very active metabolism which, in a few hr, causes a pH drop in the culture medium. This requires frequent adjustments or changes of medium to avoid necrosis. Although total confluence is not achieved, the cultures must be transferred every 8 to 10 days to avoid death of the cells.

After passage through mice, the primary cultures from the intraperitoneal nodules showed growth patterns similar to that of the original MMT cells. Small epithelial sheets and a lacy network were particularly characteristic. They differed, however, in 4 interesting properties: (a) they spread more evenly on the glass surface; (b) they had less tendency to form tissue beads and could eventually achieve confluence; (c) the pH of the culture medium remained neutral over longer periods; and (d) transfers could be spaced over longer intervals.

The same morphological variations were observed following the inoculation of MMT cells into newborn rats. Rat-passaged cells spread even better on glass than mouse-passaged cells and confluence was often achieved. Their cytoplast was clear and nongranular (Fig. 2). The pH of the culture medium was stable. After several passages into rats, the MMT cells retained their mouse characteristics as indicated by chromosome and isoenzyme analysis.

Virus Production

Mouse. Alternate passage of the MMT cells in mouse and in tissue culture have increased the production of B particles. In thin sections of MMT cells, only a few B particles (Fig. 3) were found in a small proportion of the fields observed. After passage into newborn C57BL mice, B particles were found in larger numbers and a greater proportion of fields. As shown in Table 1, there was an increase in the production of B particles in all the mouse-passaged cells well over that of the original MMT cell line. The best production was observed in the cultures derived from Passage 4 (Fig. 4).

| Transfer numbers indicate transfers only from beginning of experiment or since last animal passage. |
|---|---|---|---|---|
| Cells | Mouse passage No. | In vitro transfer No. | B particles in thin sections of cells | Whole-cell mount (% cells with B particles) |
| MMT | Original | | | |
| MMT/C57BL | 3 | 13 | ++ | 6 |
| | 4 | 13 | ++ | 25 |
| | 5 | 3 | ++ | 30 |
| | 6 | 3 | ++ | 25 |
| MMT/Af | 1 | 3 | ++ | 30 |
| MMT/A | 3 | 10 | +++ | 35 |
Passage through newborn Af and A mice also caused a higher productivity. After injection of the MMT cells in newborn Af mice, the increase of B particles in the derived cultures was noted from the very first passage. When injected into A mice, however, the MMT cells rarely developed into nodular growths; the mice usually died of unknown causes. One of those which survived for 6 weeks presented on autopsy an enlarged spleen and several nodules dispersed throughout the peritoneum. These nodules, explanted in tissue culture, gave rise to a cell line producing a consistently high yield of B particles (Fig. 5).

The whole-cell-mounting technique confirmed these results. Whereas in the original MMT line 6% of the cells were seen shedding virus, 25 to 35% produced B particles after passage in the mouse. The level of B particle production in tissue culture was found to be dependent on the time following explantation and decreased beyond the 4th or 5th transfers. The maximum budding usually occurred 4 to 7 days after explantation of the cells and on the number of transfers. The maximum budding occurred 4 to 7 days after explantation and decreased beyond the 4th or 5th transfers. However, a new passage of the cells into mouse always restored a higher budding rate. The stimulation of virus production was evidenced in two ways: (a) the percentage of cells shedding virus was more than doubled and (b) the average number of virions shed by the producing cells was increased many-fold.

B particles, readily distinguishable from other cell components by their limited size range and spacing of surface spikes, were also found in ultracentrifugation pellets of culture supernatants; only 12 ml were needed to find aggregates of viral particles (Fig. 6). An attempt was made to compare quantitatively the number of B particles released by MMT/mouse-passaged cells with that of the original MMT cell line. Culture fluid, 100 ml harvested at the end of a 7-day cultivation period from flasks seeded with the same number of cells, was collected from each cell line and processed as described in “Materials and Methods.”

Counts of the virus particles were made following the method of Watson et al. (20). As shown in Table 2, the increase in B particle production after mouse passage was about 5-fold.

The indirect method of membrane immunofluorescence also provided means for a rapid estimation of MTV antigen (Table 3). When MTV was present, the periphery of the cells appeared as a bright green fluorescent ring (Fig. 7). This ring was not observed when normal serum was substituted for the MTV antiserum or when normal cells were treated with the MTV antiserum. When tested by this method, the original MMT line showed a specific fluorescence in 20% of the cells. In contrast, the amount of fluorescent cells observed in mouse-passaged lines ranged from 35 to 75%.

The immunodiffusion method was less sensitive, but it corroborated the results obtained by other methods (Table 3).

### Table 3

<table>
<thead>
<tr>
<th>Cells</th>
<th>Passage</th>
<th>Transfer</th>
<th>Membrane fluorescence (%)</th>
<th>Immunodiffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMT</td>
<td>Original</td>
<td>13,16</td>
<td>20</td>
<td>Negative</td>
</tr>
<tr>
<td>MMT/C57BL</td>
<td>2</td>
<td>3,6</td>
<td>75</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3,11</td>
<td>5</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>35</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>11</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>MMT/Amsterdam rat</td>
<td>2</td>
<td>2</td>
<td>75</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2,8,10,26</td>
<td>75</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*Transfer numbers indicate the subculture on which membrane fluorescence or immunodiffusion tests were performed.*

### Rat

Alternate passages of the MMT cells through rats did not result in the immediate increase of B particle production (Table 4). Thin sections of the nodular growths of cultured cells showed no B particles throughout the first 3 passages.

Detection, as well as increased production, of B particles became evident only after 4 passages of the MMT cells in Amsterdam/IMR rats. In Passage 5, the B particle yield was comparable to that found in the best mouse-passaged cell line. Further, the presence of MTV antiserum or when normal cells were treated with the MTV antiserum. When tested by this method, the original MMT line showed a specific fluorescence in 20% of the cells. In contrast, the amount of fluorescent cells observed in mouse-passaged lines ranged from 35 to 75%.

### Table 4

<table>
<thead>
<tr>
<th>Cells</th>
<th>Rat passage</th>
<th>In vitro transfer</th>
<th>B particles in thin sections of cells</th>
<th>Whole-cell mount (% cells with B particles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMT/Amsterdam rat</td>
<td>1</td>
<td>4</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>18</td>
<td>+++</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4</td>
<td>+++</td>
<td>55</td>
</tr>
</tbody>
</table>
line: MMT/A-P 3 (Table 1). The morphology of the virions was characteristic of MTV with spikes on the envelope and the classical excentric nucleoid in the mature particles. This was confirmed by negative staining of whole-cell mounts (Figs. 8 and 9).

Virus production was, as in the mouse-passaged cells, dependent on the time following explantation (6), but unlike the mouse-passaged cells, it was not dependent on the number of generations in vitro. As already reported (6), active budding was usually observed until the cells began to degenerate; a high production of particles was maintained in all successive transfers. High yields of MTV antigen have been found in rat-passaged cells after 44 serial transfers and 11 months in tissue culture.

Ultracentrifugation of culture supernatants after 8, 10, and 12 serial transfers resulted in pellets rich in viral particles. A quantitative measurement of B particles released in 100 ml culture supernatant by a number of rat-passaged cells equal to that used for MMT cells and MMT/mouse-passaged cells was made. All conditions being the same, the increment of B particles in the rat-passaged cells was about 30 times the number of B particles found in the original MMT cell line (Table 2).

Clumps of virus have also been obtained from the ultracentrifugation of only 1 ml culture supernatant in a Sharp sucrose density gradient. Furthermore, a light-scattering band was obtained by density gradient centrifugation after 300-fold concentration of 200 ml MMT/Amsterdam rat-5 cell culture supernatants.

Immunofluorescence confirmed the high productivity of the rat-passaged cells by its intensity. About 80% of the cells were found to carry a specific MTV antigen (Table 5).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Passage No.</th>
<th>Transfer No.</th>
<th>Membrane fluorescence (% cells fluorescent)</th>
<th>Immunodiffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMT/Amsterdam</td>
<td>5</td>
<td>7, 12</td>
<td>80</td>
<td>Positive</td>
</tr>
<tr>
<td>rat</td>
<td></td>
<td>30, 44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMT/Amsterdam</td>
<td>6</td>
<td>7, 9, 11, 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat</td>
<td></td>
<td>9, 16</td>
<td>80</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Immunofluorescence and immunodiffusion tests on MTV cells passed in Amsterdam/IMR rats

The same anti-MTV serum as indicated in Table 3 was used. The 80% ratio of fluorescent cells between Transfers 7 and 44 in Passage 5 and between Transfers 9 and 16 in Passage 6 demonstrates the constancy of MTV antigen production.

DISCUSSION

The continuous production of B particles in a tissue culture system at a level sufficiently high to permit their isolation and study was achieved. As observed by Cardiff et al. (2), there was to date, "no adequate in vitro method for the production of this virus." Ever since its discovery by Bittner, many attempts have been made, but they have been unsuccessful mainly because of the lack of a rapid assay. In a recent report on primary cell cultures from dissociated BALB/cf3H tumors, Cardiff et al. (1) described a technique for MTV mass production in vitro. A heavy seeding of 10 to 50 X 10^6 cells/culture flask provided the release of enough B particles to be demonstrated by a light-scattering band in a sucrose density gradient.

As a technique for producing virus, primary cell cultures have a number of shortcomings. It is known that neoplastic cells have the ability to grow in multilayered masses, but primary cultures are most unpredictable. Their growth depends on a variety of factors pertaining to the donor, the quality of the tumor, and the adaptability of the cells to their environment. The virus produced, therefore, varies greatly from one culture to the next (15).

The present procedure of alternate passages of MMT cells to newborn animals and back to tissue culture has the advantage of using an already established cell line with a well-known history of MTV production (4, 18).

Original MMT cells have always shown an exclusive budding of B particles; nevertheless, C particles have been observed after passage of these cells through C57BL, A/J, and A newborn mice. We have previously reported the endemic presence of C type leukemia particles in mice and pointed out the predominance of the LV over MTV in tissue culture (8). The possibility that MMT cells should be able to produce MTV and LV simultaneously and without apparent interference is a challenging observation on which a more detailed report will be given later.

In addition to a contamination by LV, passage of MMT cells through the mouse has another drawback: the initial increase of B particles obtained by mouse passage was not maintained in serial subcultures; a repeat passage in newborn mice was necessary to raise again the number of B particles to the higher level. Since in all our attempts the total increment of B particles rose to only 4 to 5 times the amount normally produced by the original MMT cells, it appears that the mouse is a relatively poor choice as a passenger host.
By comparison, passage of MMT cells into newborn rats not only increased the production of B particles 25 to 30 times the original MMT level, but it also maintained this high production through subsequent transfers. Contamination with LV did not occur; furthermore, the stability in pH of the culture medium and the even rate of growth of the rat-passaged cells opened the possibility of producing large quantities of MTV of greater purity than could be obtained from milk or tumor extracts.

The stimulated virus production obtained by the alternate passages of the mouse tumor cells in rats and in tissue culture might eventually serve as a model system to increase or reveal the presence of a virus in human tumors.

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