In Vitro Production of Mouse Mammary Tumor Virus in a Mouse Mammary Tumor Ascsites Line

(RNA-directed DNA polymerase/70S RNA/viral antigens/DNA-RNA hybridization)

JAPA KEYDAR*, ZVEE GILEAD*, JACOB R. HARTMAN*, AND YEHUDA BEN-SHAUL†

*Department of Microbiology and †The Laboratory for Electron Microscopy, University of Tel-Aviv, Ramat-Aviv, Israel

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ABSTRACT An ascites line derived from a spontaneous mouse mammary carcinoma produces, on explantation and cultivation in vitro, large amounts of oncornavirus particles. The biochemical, biophysical, and electron microscopic characteristics of the virions are described. Molecular hybridization and immunological methods identify these virions as mouse mammary tumor virus.

Human tumors contain some RNA sequences homologous to those of murine oncornaviruses (1–5). Radioactive DNAs synthesized on RNA of murine mammary tumor virus (MMTV) and murine leukemia virus (RLV) as templates with RNA-directed DNA polymerase were used as probes in DNA–RNA hybridizations. Human breast carcinomas contain RNA homologous to that of MMTV (1, 2), whereas human leukemias (3), sarcomas (4), and lymphomas (5) contain RNA homologous to that of RLV. Furthermore, it was shown that those related RNA sequences in the human tumors were associated with particles possessing 70S RNA, an RNA-directed DNA polymerase, and a buoyant density of 1.15–1.19 g/cm³ in sucrose gradients (6, 7).

We recently described the establishment of human embryonic cell lines producing oncornavirus that were obtained either by infection with milk from breast-cancer patients or by cocultivation with human breast carcinomas (8). The oncornavirions in these cultures can now be identified by hybridization with appropriate DNA probes. For this purpose it would be highly advantageous to have a mouse line that produces large quantities of virus. This type of line would, of course, also be of value for comparative studies with our human lines. We have developed such a mouse line that produces large amounts of MMTV. This line, an ascitic line designated TA3 (9), and its virus are the subject of this paper.

MATERIALS AND METHODS

Cells and Virus. TA3 is a spontaneous mammary tumor that arose in a A/HeHa mouse in the laboratory of Hauschka (9). From 1949 to 1951 it was passed for 16 generations in mice as a solid tumor and then made into an ascites line by Klein (10) and kept in A/Sn mice. In our laboratory it has been propagated serially in A/J mice. The mice were injected intraperitoneally with 10⁶ cells per mouse, and the ascitic fluid containing 1 to 2 × 10⁶ cells per mouse was harvested 1 week later. The line is highly lethal and kills mice in 8–10 days after injection of 10⁶ cells. It was therefore transferred weekly into new hosts.

Two types of in vitro cultures were established from the explanted ascites cells: short-term and long-term stable lines. Both types of cultures were maintained in Roux bottles containing 100 ml of RPMI-1640 medium which was supplemented with 10% calf serum, 0.2 U/ml of insulin (Wellcome), and 20 µg/ml of hydrocortisone hemisuccinate (8, 11). For the long-term cultures, the cells from the ascitic fluid were seeded at 10⁶ cells per ml. These cells adhered only weakly to the glass, and were continuously depleted from the cultures because of the daily medium changes. These cultures did not survive beyond the tenth day after explantation. Long-term stable lines could, however, be obtained from these cultures when the medium was supplemented with fetal-calf serum in addition to new-born calf serum. Cells in these latter cultures adhered strongly to the glass and replicated rapidly. Consequently, they were subcultured by EDTA–trypsin treatment (8) every 3–4 days. Cells from the long-term cultures remained lethal to mice even after many culture transfers. The medium in both the short-term and long-term cultures was changed daily. Harvested medium, clarified by sedimentation at 7700 × g for 15 min, was stored at −70°C. Such clarified supernatants served as our routine source of virus particles.

Electron Microscopy. TA3 cells were prepared for electron microscopy by the procedure of Calafat (12). Cells were fixed with 1% osmium tetroxide in malonate buffer for 20 min, dehydrated, and embedded in Epon. Thin sections on grids were stained overnight with a saturated solution of uranyl acetate in 30% alcohol, post-stained for 15 min with 0.5% lead citrate, and observed in a Jem T-7 electron microscope. Viral preparations were also negatively stained on grids with 2% uranyl acetate.

Concentration and Purification of Virus. Particles in the culture medium of TA3 cells were sedimented onto 100% glycerol cushions at 90,000 × g for 50 min. The particles were then collected, put on top of a 20–60% sucrose gradient in TNE buffer [10 mM Tris-HCl–150 mM NaCl–2 mM EDTA–3 mM dithiothreitol (pH 8.3)] and sedimented to equilibrium at 90,000 × g (4 hr) or 53,000 × g (overnight) in an SW-27 rotor of a Spinco ultracentrifuge. Fractions were collected and their density was determined by refractometry. Particles sedimenting at the density region of 1.15–1.19 g/cm³ were pooled and pelleted at 90,000 × g for 50 min. All operations were done at 4°C.

Purification of Virus Cores. The cores were isolated essentially as described by Bader et al. (13). Virus was pelleted from the 1.15 to 1.19 g/cm³ density region of sucrose gradients and suspended in TE buffer [50 mM Tris–2 mM EDTA–3
mM dithiothreitol (pH 8.3)); the nonionic detergent NP-40 (Shell) was added to a final concentration of 0.5%. After 15 min at 4 °, the preparation was layered on top of a 20–60% sucrose gradient in TE buffer and sedimented to equilibrium. Sedimentation was at 53,000 × g overnight in an SW 27.1 rotor. Fractions were collected and the absorbance at 260 nm was measured. Material from the single band observed was pelleted at 90,000 × g for 15 min.

**RNA-Directed DNA Polymerase Activity** in the virions was measured either with endogenous RNA template or with exogenous poly(dT)·poly(rA) template by described procedures (8, 14, 15). The virions were disrupted with 0.33% of NP-40 before assay.

**Serological Procedures.** The micro-complement fixation technique of Sever (16) was used with 1.5 units of complement and 2 or more units of antibody. Purified virions and isolated cores were frozen and thawed five times and sonicated three times for 15 sec before complement fixation assay.

**DNA–RNA Hybridization.** The homology between 70S viral RNA extracted from the virions in the TA3 cells and radioactive DNAs synthesized on MMTV and RLV templates with RNA-directed DNA polymerase was assayed in a DNA–RNA hybridization reaction. Synthesis of the radioactive DNA product with virions purified from the TA3 cells, extraction of the 70S viral RNA from these virions, and its isolation in glycerol gradients and analysis of the products of the annealing reaction in CsSO₄, was performed by methods of Spiegelman and his coworkers (1, 6, 15, 17). Radioactive RLV DNA product and radioactive MMTV DNA product (synthesized with particles purified from the milk of Paris RIII, as well as unlabeled 70S RNAs from these viruses, were obtained from Dr. S. Spiegelman.

**RESULTS**

**Detection of Oncornavirions in TA3 Cells.** Electron microscopic observation of thin sections of TA3 cells disclosed a great abundance of budding virions and also intracytoplasmic bodies resembling A-type particles. The latter are immature precursors of complete B-type particles of MMTV, or of complete C-type particles of leukemia and sarcoma oncorna-viruses (18). A preliminary electron microscopic study indicated that cells obtained immediately after explantation (from the ascitic fluid) contain only A-type particles and no mature budding particles. However, after in vitro culture in hormone-containing medium (11), mature budding particles were seen. They were scarce on the first and second day in culture but appeared later in great numbers, with a peak count at the fourth and fifth day after explantation. The long-term established cultures showed, by contrast, a steady high level of budding virions. Plate 1 demonstrates the electron microscopic morphology of the virions in short-term cultures, on the fourth day after explantation. Part A shows part of a cell with numerous intracytoplasmic immature particles and
some budding particles; B shows an intercellular space with mature virus particles budding from villi; C shows a single enlarged particle demonstrating the finer details of the virion.

A preliminary identification of the particles as oncornavirions was made by isopycnic banding of particulate material from the culture media in a 20–60% sucrose gradient. The fractions from the gradient were measured for absorption at 260 nm, density, and presence of [3H]dTMP-incorporating activity in an RNA-directed DNA polymerase assay with added poly(dT)-poly(rA) as template (8, 14). A band of material absorbing at 260 nm, characteristic of oncornavirus density (1.15–1.19 g/cm³) with a coincident [3H]dTMP-incorporating activity, was observed (Fig. 1).

Particles purified by banding in the 1.15–1.19 g/cm³ density region of sucrose gradients were pelleted (90,000 × g, 50 min) and assayed in an RNA-directed DNA polymerase assay with their endogenous RNA template. [3H]dTMP-incorporating activity was identified as an RNA-directed DNA polymerase activity by virtue of its dependence on endogenous RNA. Activity was abolished by pretreatment of the disrupted particles with a mixture of pancreatic and T1 ribonucleases (Fig. 2). The kinetics of the reaction showed a steady enzyme activity until the fourth hr. Use of 0.1–0.33% NP-40 for disruption of the virions gave similar reaction kinetics, whereas 0.5 and 0.8% were slightly inhibitory.

The virions contain a characteristic oncornaviral 70S RNA template, as shown in an experiment with virions collected from a culture labeled for 24 hr with [3H]uridine. Labeled virions collected from the 1.15 to 1.19 g/cm³ region of a sucrose gradient were extracted with Na dodecyl sulfate and phenol–cresol (8, 15) and the RNA was subjected to velocity sedimentation in a 5–20% sucrose gradient (Fig. 3).

The DNA product synthesized in a reaction mixture containing only disrupted virus particles and deoxyribonucleoside triphosphate precursors efficiently hybridized to 70S RNA from the same particles. Analysis of the annealed products in a CsCl gradient gave a profile similar to that in Fig. 5A.

Identification of Oncornavirions in TA3 Cells. The virions were analyzed by complement-fixation tests with reference antisera to murine type-B and type-C oncornaiviruses. Virus banded once in 20–60% sucrose gradients as well as twice-banded virus and isolated cores were assayed. The cores were obtained from virus treated with NP-40 (0.5%) and banded in a 20–60% sucrose gradient (Fig. 4). The electron-microscopic morphology of the twice-banded virions and the purified cores is demonstrated in Plate 2. The nucleoid or core of the oncornaiviruses is the site of the group-specific (gs) antigens (19–23). It lacks the external lipoprotein membrane which contains host elements and is thus devoid of mouse antigens. The cores can react with group-specific antisera and classify the virions as belonging to the MMTV group, or leukemia-sarcoma group. Results obtained with the purified virus and cores in the complement fixation test are summarized in Table
Fig. 3. Detection of 70S RNA template in purified virions. A TA3 culture in a Roux bottle was labeled for 24 hr with 30 μCi/ml of [3H]uridine (14.3 Ci/mmol) and medium was collected at the end of this period. Chase medium was added twice during the next 48 hr and was also collected. Labeled virus particles were purified as described in the legend to Fig. 1. The virions from the 1.15 to 1.19 g/cm² density region were extracted by the Na dodecyl sulfate–phenol–creosol method and the RNA was subjected to velocity sedimentation in a 5–20% sucrose gradient. An external marker of 3H-labeled λ DNA marker (32 S) was sedimented in a separate tube.

1. They identify the virions in the TA3 cells as MMTV and indicate that the cores are indeed the antigens of choice since they are free of reactivity with antiserum to mouse-cell components.

The virus in the TA3 cells was further identified as MMTV in DNA–RNA hybridization reactions. Radioactive DNA synthesized in MMTV particles from the milk of Paris RIII mice (a strain with a high incidence of mammary tumors) annealed with excess 70S RNA from TA3 virions. Analysis of the annealing products in CsSO₄ (Fig. 5A) demonstrated a clear shift of most of the labeled DNA to the RNA position in the gradient. No such shift was observed when the 70S RNA from the TA3 virions was reacted with 3H-labeled RLV RNA (Fig. 5B), whereas 70S RLV RNA hybridized with its homologous DNA and the RNA–DNA hybrid formed shifted to the RNA position of the CsSO₄ gradient (Fig. 5C). These studies clearly indicate major homology between particles from the TA3 cell line and MMTV particles from milk.

**TABLE 1. Complement fixation tests with various reference antisera and purified virus and cores**

<table>
<thead>
<tr>
<th>Antigen titer* obtained with the following antisera</th>
<th>Pig anti-MLV†</th>
<th>Goat anti-MLV (disrupted)‡</th>
<th>Goat anti-MLV lymphocytes§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>Rabbit</td>
<td>Goat</td>
<td>Goat</td>
</tr>
<tr>
<td>Once-banded virus</td>
<td>1:128</td>
<td>1:16</td>
<td>1:16</td>
</tr>
<tr>
<td>Twice-banded virus</td>
<td>1:32–1:64</td>
<td>1:4</td>
<td>1:2</td>
</tr>
<tr>
<td>Cores</td>
<td>1:16–1:32</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
</tr>
</tbody>
</table>

* Last antigen dilution that gave complete fixation.
† Obtained from Dr. J. Verna (Meloy Laboratories). Rabbits were injected with purified B-type particles from the milk of C3H mice in complete Freund's adjuvant.
‡ Obtained from National Cancer Institute, Special Virus Cancer Program (SVCP). A pig was injected with purified disrupted Moloney leukemia virus in complete Freund's adjuvant. This antiserum gives two immunodiffusion lines against tumor suspensions from Gross tumors, Moloney sarcoma tumors, AKR tumors, and Tween–ether-disrupted Moloney leukemia virus.
§ Obtained from the SVCP. A goat was injected with purified intact Moloney leukemia virus. This antiserum gives two immunodiffusion lines with Tween–ether-disrupted MLV and one line with intact MLV. It was positive in the complement fixation test with MLV at >1:20.

**FIG. 4. Isolation of cores.** Virus was purified as described in legend to Fig. 1 and pelleted at 90,000 X g for 50 min; the pellet was dissolved in TE buffer. Two-thirds of the pelleted virus (1.2 mg of protein) was treated for 15 min at 4°C with a final concentration of 0.5% NP-40, and the other third (0.6 mg) was not treated. Both the untreated (A) and the disrupted (B) virus were sedimented to equilibrium in a 20–60% sucrose gradient in TE buffer for 15 hr at 33,000 X g in an SW 27-1 rotor.

**Plate 2.** Electronmicrographs of negatively stained preparations of: (A) twice-banded virions; (B) purified cores. Magnification for A and B, X 192,500.
The sole source of MMTV for immunological and biochemical studies at present are particles derived from mammary tumor tissues or from the milk from mouse strains with a high incidence of tumors. However, several attempts to obtain in vitro production of MMTV have been made. Thus, organ cultures or primary tissue cultures of mouse mammary tumors were made (24). The first yielded low levels of virus whereas the latter gave considerable yields but were short-lived. Several long-term mouse mammary tissue culture lines were also established. However, they produced an abundance of B-type particles early after explantation, but this production generally subsided after 1-6 months in vitro (25). Another serious shortcoming of tissue-cultured mouse mammary tumors, or milk from mice with a high incidence of mammary tumors, is the wide-spread contamination of such sources with C-type particles, since mice are hosts to sarcoma and leukemia viruses (26, 27). Lasfargues et al. (28) described a stable cell line, designated MMT, that was developed by Dr. J. Sykes (29) from a spontaneous mammary tumor. This line exhibited a continuous low level of virus production when examined by electron microscopy. Virus production could be greatly increased by alternate passages from tissue culture to animals (as solid tumors) and back (28). However, such passage through animals is open to contamination with C-type particles during animal passage.

Several mouse ascites lines derived from spontaneous mouse mammary tumors are known. Notable among these are the TA3 (9), the Ehrlich, and the MM102 ascites lines (30). These use the peritoneal cavity of mice as a tissue-culture flask and yield a large number of disseperse, homogeneous tumor cells 1 week after inoculation. The TA3 line produces a great abundance of MTV both immediately after explantation and after long-term culture. It should be stressed that the yield of virus during the first and second day after explantation is low and increases to high levels only after 3-4 days in cultures. The

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