Experimental Infection of a Cat Kidney Cell Line with the Mouse Mammary Tumor Virus

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SUMMARY

A cat kidney cell line, CRFK-F2, was successfully inoculated in suspension and in monolayer culture with a purified mouse mammary tumor virus derived from RIII milk. The virus produced by the infected cells was identified by immunofluorescence, electron microscopy, and RNA-directed DNA polymerase assays; it was a B-type virion that did not cross-react with mouse or feline leukemia-sarcoma viruses, had spikes on its envelope, and had a RNA-directed DNA polymerase reaction that was typical of mouse mammary tumor virus. The producing cells were identified as cat cells by chromosome number, cytotoxic assays, and isoenzyme migratory patterns. A standardized method for the in vitro inoculation of cat cells is described that presently permits highly reproducible results. For the first time, the mouse mammary tumor virus is seen replicating in cells from another species, thus offering an opportunity to study the kinetics of infection of that virus.

INTRODUCTION

Of all the murine oncogenic viruses studied in the past 10 years, the MuMTV\(^1\) has been the most difficult to understand because of its inability to infect species other than the mouse or to infect homologous, known susceptible target cells in tissue culture. As a result, there is to date no simple in vitro assay to evaluate the infective and transforming properties of this virus.

Recent evidence (7) for MuMTV replication in a cat kidney cell line (CRFK-F2) might offer such a possibility. The confirmed susceptibility of cat kidney cells to MuMTV has definite advantages; because of their non-mouse origin, cat kidney cells are free from endogenous murine viruses, and they also might be susceptible to infection by mammary tumor viruses from many other species.

Earlier difficulties met in repeating this type of experimental infection were of a technical nature. Since then, however, in vitro infections have been accomplished under a variety of conditions; enhancement of MuMTV release by the infected cells was obtained by dexamethasone stimulation and the biochemical detection of a RDDP was correlated with the increase of virus. This report describes these complementary experiments.

MATERIALS AND METHODS

The cat kidney cells used in our studies came from a stock cell line, CRFK, established by Crandell et al. (1) in 1964. A subline, CRFK-F2, was obtained, within the NIH virus-cancer program, from the Naval Biomedical Research Laboratory through the courtesy of Dr. W. A. Nelson-Rees, Oakland, Calif.

The CRFK cells are epithelioid and double their population in 40 to 48 hr in HE-MEM supplemented with 10% fetal calf serum and 10 \(\mu\)g insulin per ml.

HE-MEM and fetal calf serum were obtained from Flow Laboratories, Rockville, Md. Insulin, isolated from bovine pancreas, was purchased from Schwarz/Mann, Orangeburg, N. Y. A concentration of 10 \(\mu\)g insulin per ml is equivalent to 0.23 IU. Dexamethasone, obtained from Sigma Chemical Co., St. Louis, Mo., and Gentamicin, from Lederle Laboratories, Pearl River, N. Y., and Gamentin, from the Schering Corp., Union, N. J., were incorporated into the culture medium at a concentration of 10 \(\mu\)g/ml. Achromycin, from Lederle Laboratories, Rockville, Md., was added to the culture medium at a concentration of 10 \(\mu\)g/ml.

Preparation of the Inoculum. The MuMTV used to infect the cells was routinely derived from 4 ml of crude milk collected by suction from about 8 lactating RIII mice. Milks from the 2nd and later lactations were pooled and stored at 4° with 500 \(\mu\)g/ml Gentamicin to prevent bacterial contamination. On the day of use, the pooled milk was skimmed by a 5-min centrifugation at 5,000 rpm in a Spinco 25.1 rotor and then pelletized by another centrifugation at 21,000 rpm for 1 hr in the same rotor. The pellet was resuspended overnight at 4° in 0.2 ml of a phosphate-buffered saline containing 0.46 g monobasic sodium phosphate, 4.08 g anhydrous dibasic sodium phosphate, and 8.78 g sodium chloride per liter of distilled water. This virus suspension which contained about 1.5 mg viral protein was layered on a 25% Ficoll gradient (Pharmacia, Uppsala, Sweden) and spun for 1 hr at 32,000 rpm in the Spinco 50.1 rotor. A light-scattering band observed at the 1.052-g/ml density level (equilibrium not reached) was then removed with a capillary pipet, reconstituted to the original 4-ml volume with HE-MEM, and filtered through a 0.45-\(\mu\)m Millipore membrane coated with PVP. PVP, an inert, nontoxic or-

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ganic polymer, has been found to reduce or even eliminate virus adsorption by Millipore membranes (15). The PVP-K-90 that we used had an average molecular weight of 3.6 × 10^6 and was obtained from the General Aniline and Film (GAF) Corp., New York, N. Y. Prefiltration of a 1% solution of the polymer in phosphate-buffered saline provided an efficient coating that ensured bacterial sterility without loss of virus.

Inoculation. Trypsinized CRFK-F2 cells were resuspended in HE-MEM containing DEAE-dextran (Pharmacia), 25 μg/ml, and incubated for 30 min at 37°C in a water bath. The cells were then washed twice in HE-MEM and resuspended in the inoculum at a concentration of 10^6 cells per ml. After 1 hour of incubation at 37°C with mild intermittent agitation, the suspension was diluted with 4 times its volume of complete culture medium and distributed in culture flasks. Control cells treated in the same way were exposed to a concentration of Ficoll comparable to that of the virus inoculum.

Recently, we have been using Polybrene (Aldrich Chemical Co., Milwaukee, Wis.) instead of dextran. Polybrene is nontoxic to cells and can be added directly to the inoculum up to a final concentration of 4 μg/ml. It can also be introduced into the culture medium without observable side effects to the cell growth or metabolism. This simplified the inoculation procedure since the cells do not have to go through the pretreatment and double wash required with dextran.

Detection. The cells were screened for virus production by membrane immunofluorescence, electron microscopy, and reverse transcription assays.

For immunofluorescence we used a rabbit antiserum prepared against purified RIII virions (6). The indirect method described by Klein et al. (5) for live, freshly trypsinized cells was applied. This technique was found more reliable than fluorescence on fixed cells in which modified permeability of the plasma membrane normally leads to nonspecific reactions. The MuMTV antiserum was prepared and absorbed as specified by Nowinski et al. (11); labeling was obtained with a commercial fluorescein-tagged anti-rabbit γ-globulin. All positive cells presented a bright continuous ring of peripheral fluorescence.

The search for physical virions was made by electron microscopy in: (a) high-speed centrifugation pellets of culture supernatants negatively stained with sodium phosphotungstate; (b) whole-cell preparations prepared by the method of Kramarsky et al. (6); and (c) thin sections of scraped cells fixed in glutaraldehyde and osmium tetroxide, then embedded in Epon-Araldite.

The RDDP assays were performed by monitoring poly(rC)-oligo(dG)-directed DNA polymerase activity in 100 μl concentrates of culture supernatants. RDDP activity was measured in terms of [3H]-dGTP incorporation as acid-precipitable counts. Since this value is proportional to the amount of virus released by the cells, one can estimate with fair accuracy the level of virus production. The dependence of the reaction on Mg²⁺ or Mn²⁺ ions further indicated whether the virus produced was MuMTV or murine leukemia virus (2).

RESULTS

The CRFK line is epithelioid and has a uniform rate of proliferation. Inoculation with the mouse mammary tumor virus did not change significantly the rate of growth; furthermore, no morphological variation or modulation that could be interpreted as a signal of infection was observed even several months after inoculation.

Immunofluorescence. The 1st sign of virus replication was demonstrated by a routine survey of the cells by membrane immunofluorescence. About 1 month postinoculation 10 to 15% of the cells exposed to a properly absorbed MuMTV antiserum showed the characteristic peripheral ring indicating the presence of a specific surface antigen. The specificity of this reaction was demonstrated by a lack of fluorescence from sham-inoculated cells exposed to the same antiserum. A normal rabbit serum absorbed in the same conditions as the antiserum failed to elicit fluorescence in both experimental and control cells. The ever-present possibility that a cat virus, perhaps from the leukemia family, might be produced at the cell surface and cross-react antigenically with MuMTV was tested with antiserum against murine leukemia virus, feline leukemia virus, and RD-114. The last 2 antiserum were secured through the virus

Table 1

<table>
<thead>
<tr>
<th>Antisera</th>
<th>MuMTV</th>
<th>MuLV</th>
<th>FeLV</th>
<th>RD-114</th>
<th>Mouse</th>
<th>NRS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRFK-1 (control)</td>
<td>+ + (25)</td>
<td>-</td>
<td>-</td>
<td>+ (10–15)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BALB/cf3H</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ (10–15)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gardner mouse testicular tumor</td>
<td>++++ (100)</td>
<td>-</td>
<td>-</td>
<td>++++ (100)</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

* NRS, normal rabbit serum; MuLV, murine leukemia virus; FeLV, feline leukemia virus; RD-114, endogenous feline virus; Mouse, mouse embryonic tissues antiserum; NT, not tested.
that no cross-reactivity existed between RD-114 and of response of BALB/cfC3H cells to that antiserum suggests that 114 antiserum reacted with 10 to 15% of the cells of the CRFK stock line as well as with the experimental. The MuMTV nature of the antigen detected at the surface of the CRFK inoculated cells was therefore highly probable. Replication of the virus became evident as the number of fluorescing cells increased up to 30% in the following 6 months of cultivation.

**Electron Microscopy.** The finding of intact virions by electron microscopy confirmed the immunofluorescence data. Negatively stained high-speed centrifugation pellets of culture supernatants contained virus structures morphologically comparable to the MuMTV. Spikes on the external envelope of the pelleted virus bodies were particularly revealing since the type C virions usually have a smooth surface. The persistence of spiked virions in culture supernatants 10 months after inoculation could not be logically attributed to a carry-over of residual virus; rather it was strongly indicative of virus replication.

Production of MuMTV at the level of the cell membrane was seen in whole-cell mount preparations of single-cell suspensions. The virions that formed at the tip of microvilli were of the B-type morphology as indicated by the protrusion of spikes well defined by negative staining. The B particles proved to be relatively numerous in electron microscope scans of thin sections (Fig. 1). The diameter of the budding virus was the same (100 nm) as for MuMTV. Even though external spikes seen in thin sections were not as clearly defined as in mouse cell cultures (Fig. 2), the finding of mature particles with eccentric nucleoids (Fig. 3) left little doubt as to their identity.

**RDDP.** A RDDP that could direct DNA synthesis on oligo(dG)-poly(rC) template was detected in the culture supernatant of the infected cat cells 3 months after their inoculation with RIII virus. The dependence of that reaction on magnesium supported the MuMTV nature of the virus.

**Effect of Dexamethasone.** Following reports that dexamethasone stimulated MuMTV expression in tissue culture (3, 12), 10 μg of this synthetic corticoid were added per ml of culture medium. A search for virus release was made 48 to 72 hr later. The immunofluorescence response of steroid-stimulated versus nonstimulated cells was readily significant with an increase from 25 to 95% fluorescing cells (Table 2). This increase correlated with a corresponding elevation in the amount of RDDP detected in the culture supernatants. Using an RNase-resistant methylated template, poly(rCm)-oligo(dG), a 6-fold increase in the amount of MuMTV released by the cat cells was demonstrated. Since MuMTV displays a stringent requirement for Mn²⁺ with the poly(rCm)-oligo(dG) template (A. S. Dion, unpublished results), cation preference was assayed with poly(rC)-oligo(dG) in the presence of either 10 mM Mg²⁺ or 0.4 mM Mn²⁺. As shown in Table 2, the latter template-directed synthesis was considerably greater in the presence of Mg²⁺ and was typical of B-type MuMTV (2).

In a repeat experiment (CRFK 17) dexamethasone was added to the culture medium 10 days after inoculation with RIII-MuMTV. Tests for immunofluorescence on Day 12 showed about 20% positive versus 2 to 5% for cells not exposed to the steroid. Thus, the dexamethasone seemed to hasten virus production.

**Bioassay.** Dexamethasone-treated, virus-producing cells were trypsinized and inoculated i.p. into 6- to 8-week-old C57BL mice in amounts of 3 × 10⁶ cells/mouse. The cells did not grow, as could be expected from cells placed in a nonhistocompatible organism. However, the milk from 9 of 20 inoculated mice contained MuMTV antigen that could be detected by gel immunodiffusion 5 months after injection. One of these mice has developed a mammary tumor in the inguinal region at 9 months of age, which is significant since C57BL mice normally never develop mammary tumors.

**DISCUSSION**

Because of the cumulative evidence that MuMTV was infective in vitro and replicated in cat kidney cells, it became imperative to ascertain (a) whether or not such an experimental system was reproducible and (b) whether or not the virus-producing cells were mouse cells accidentally introduced in the cat cultures with the inoculum or at any other time with the feeding solution.

**Reproducibility.** In our early attempts (Table 3), the major obstacle to reproducibility was bacterial contamination. Even though 500 μg of Gentamicin were added to each ml of crude milk, contaminations occurred because of technical difficulties met in attempting to sterilize the various parts of the gradient maker. This was compounded by the near impossibility in maintaining rigorous aseptic procedures throughout the various steps of virus purification. Filtration of the final product through an uncoated 0.45-μm Millipore membrane resulted in the adsorption of most of the virus particles and the final loss of infectivity of the inoculum.

To obviate such difficulties, it is beneficial to: (a) process milk specimens within 24 hr following their harvest; high-speed pellets from skimmed milks are prepared immediately after collection and are layered on the Ficoll gradient early the following day; and (b) coat the Millipore membrane with PVP as described in "Materials and Methods." The purified milk fraction can then be sterilized by filtration without loss of infectivity. Another determining factor has been the use of HE-MEM at pH 7.2 to 7.4 to dilute the purified virus fraction and to serve as the basic ionic solution for the culture medium. In independent experiments, it has been found that the production of MuMTV by mouse tumor cells was greater in HE-MEM than in Roswell Park Memorial Institute Medium 1640. Except for cell inoculation, the incubation temperature of the cultures was maintained at 35–36°.

As shown in Table 4, every attempt made under these new conditions was successful. A large quantity of virus appears to be required to infect a relatively small number of cells; however, if the technical details mentioned above are re-
Table 2

Effect of dexamethasone on the release of MuMTV by CRFK experimentally infected in vitro

<table>
<thead>
<tr>
<th>CRFK cells</th>
<th>Dexamethasone (µg/ml)</th>
<th>Membrane immunofluorescence (% positive cells)</th>
<th>RDDP(^a)</th>
<th>Cation</th>
<th>Poly(rC): Poly(rG) oligo(dG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control EtOH(^b)</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
<td>Mn(^{2+})</td>
<td></td>
</tr>
<tr>
<td>Control dexamethasone</td>
<td>10</td>
<td>0</td>
<td>0.13</td>
<td>Mn(^{2+})</td>
<td></td>
</tr>
<tr>
<td>Infected EtOH</td>
<td>0</td>
<td>++(25)</td>
<td>1.95</td>
<td>Mn(^{2+})</td>
<td></td>
</tr>
<tr>
<td>Infected dexamethasone</td>
<td>0</td>
<td>+++++ (95)</td>
<td>11.35</td>
<td>Mn(^{2+})</td>
<td></td>
</tr>
</tbody>
</table>

* RDDP activity expressed in pmoles [3H]dGMP incorporated per 30 min.
* Ethyl alcohol in 0.5% concentration as used to dissolve dexamethasone.

Table 3

Early attempts to inoculate CRFK cells with RIII-MuMTV in tissue culture

In each reported experiment, control cultures were treated and sham-inoculated with a gradient fraction without virus. In Experiment 10, we used a high-speed centrifugation pellet from 60 ml tissue culture supernatant collected from a BALB/cfC3H cell line. This cell line, a high MuMTV producer, had the advantage of providing a sterile inoculum. Negative results were recorded after 5 months of cultivation.

Table 4

Effect of modified and standardized techniques of inoculation of CRFK cells with RIII-MuMTV

Beginning with Experiment 12, all inoculations were done with purified virus from 4 ml of milk to infect 4 x 10\(^6\) cells. Detection was done mainly by membrane immunofluorescence 3 weeks after infection and following stimulation with 10 µg dexamethasone per ml of culture medium, 72 hr prior to the fluorescence test.

In a recent experiment, primary kidney cells obtained from 50-day-old cat embryos were inoculated on their 1st passage with RIII-MuMTV in the same manner as the CRFK cells. Two weeks later, the primary cells were stimulated with dexamethasone and then were tested by immunofluorescence for MuMTV surface antigens. About 20% of the cells were positive, thus demonstrating the efficiency of this in vitro method.

Certification of Cat Cells. The replication of a recognized mouse virus in cells from another species was startlingly expected, this experimental system can be highly reproducible.
enough to require a stringent scrutiny of the virus-producing cells. Long-established cell lines routinely carried in laboratories are known eventually to invade other cultures and in time to overcome them (10). To ascertain the feline origin of the infected cells, a number of tests were performed and the cumulative evidence removed all doubts as to the nature of the infected cells.

**Chromosomes.** The average chromosome number in the cells of our 1st successful infection was 36 to 38 evaluated from 30 mitoses. More than 1 year later, spot-checks of mitotic figures show the same number, which agrees with the chromosome number in cat cells and with the karyogram of the original CRFK-F2 cell line.

**Immunology.** An antiserum prepared in the rabbit against mouse embryonic cells did not react by indirect immunofluorescence with the MuMTV-producing cat cells but gave a 100% positive response with mouse tumor cells (Table 1). Their nonidentity with mouse cells was further confirmed by a high viability index determined by trypan blue exclusion test; no significant toxic effect was found after a 48-hr exposure to that antiserum.

**Isozyme Analysis.** The electrophoretic migration pattern, on acrylamide gel, of genetically determined enzymes is also a helpful adjunct system to verify the species of origin of a cell line (4). Extracts from cat embryo cells, stock CRFK-F2, control, and MuMTV-inoculated CRFK cells were therefore tested comparatively for LDH, glucose-6-phosphate dehydrogenase, and malic dehydrogenase with cell extracts from other species.

As shown in Chart 1, the embryonic cat cells produced multiple LDH bands that migrated at the same rate as the bands from the CRFK-F2 stock and that of control and infected CRFK cells. These were distinct from the LDH patterns of human (HeLa, Gm 183, and Gm 202), mouse, rat, and Syrian hamster cells. Significant differences were also observed with glucose-6-phosphate dehydrogenase and malic dehydrogenase. Together with the rest of our characterization data, the isozyme results clearly indicated that the MuMTV-infected CRFK cells were not of human, rat, mouse, or Syrian hamster origin, but unquestionably feline.

**CONCLUSION**

For the 1st time, evidence is presented that MuMTV, like other oncogenic viruses, can infect cells in tissue culture. The method of inoculation described was developed to provide an easy reproducibility of the inoculation process.

The sensitivity of cat kidney cells to MuMTV might not be unique, but it already permits evaluation of the importance of several parameters, one of which is the high multiplicity of virus required. The mouse mammary tumor virion as we know it by electron microscopy was, up to the present, confined to the mouse and, in spite of an exhaustive search, was never reported in other species except perhaps human milk (9). Its replication in cat cells will open new speculations as to its epidemiology.

The response of cat kidney cells to MuMTV is rapid; detection can be made 10 to 15 days after inoculation if the cells are stimulated with dexamethasone. The stimulation of MuMTV synthesis by this glucocorticoid appears to be specific and closely regulated by transcriptional controls (13). This sensitivity might be of use in the development of an *in vitro* titration assay which, thus far, was delayed because of the lack of morphological changes in the infected cells.

**ACKNOWLEDGMENTS**

The authors wish to thank Ann Flacco, Harold Ott, and John Lasfargues for their much appreciated contribution to the electron microscopy and preparation of the illustrative material.

**REFERENCES**


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**Fig. 1.** Thin section of CRFK cells stimulated with dexamethasone (10 μg/ml) 1 year after inoculation with MuMTV. The virions that form at the tip of the microvilli have a complete nucleoid, which is a most common feature of B-type virus particles. Electron micrograph, × 50,000.

**Fig. 2.** Section of a virion budding from a CRFK-infected cell. The external envelope suggests the presence of spikes. Electron micrograph, × 75,000.

**Fig. 3.** Section of a mature B particle from CRFK-infected cells, Electron micrograph, × 75,000.
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