Mouse Leukemia Virus Growth in Mouse Cells Contaminated with Mycoplasma

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SUMMARY

The growth of a mouse leukemia virus in an established mouse cell line was examined after the line became contaminated with an unidentified Mycoplasma species. The contaminated cultures grew well in small plastic cultures dishes, but they could not be propagated in larger roller bottles unless the growth medium was changed frequently. Cells from Mycoplasma-contaminated and Mycoplasma-free cultures were exposed to \(^3\)H-labeled uridine for 24 hr. Culture fluids were harvested 2 or 24 hr after labeling and purified by centrifugation through discontinuous sucrose gradients. Considerably less uridine-\(^3\)H-labeled virus was recovered from supernatant fluids of Mycoplasma-contaminated cultures than from Mycoplasma-free cultures. Equilibrium sedimentation in sucrose gradients of uridine-\(^3\)H-labeled material from culture supernatants of contaminated cultures produced \(^3\)H peaks at buoyant densities of 1.20 to 1.24 and 1.16 to 1.18 g/ml. Virus titers in culture fluids from Mycoplasma-contaminated cultures were greatly reduced as judged from viral interference tests. The viral RNA was degraded to low-molecular-weight species when virions were harvested 2 to 24 hr after labeling of Mycoplasma-contaminated cultures.

INTRODUCTION

During studies on the RNA of a MuLV, we noticed a gradual decrease in the amount of radioactively labeled virus that could be recovered from the culture fluids of a virus-producing mouse cell line (5). Supernatant fluids from certain subcultures yielded only a small radioactive peak in the buoyant density range characteristic of oncornaviruses (1.16 to 1.18 g/ml) while a larger fraction sedimented to the buoyant density range characteristic of oncornaviruses (1.16 to 1.18 g/ml). Virus fluids were harvested 2 or 24 hr after labeling and purified by centrifugation through discontinuous or linear sucrose gradients (5, 8, 21). Considerably less uridine-\(^3\)H-labeled virus was recovered from supernatant fluids of Mycoplasma-contaminated cultures than from Mycoplasma-free cultures. Equilibrium sedimentation in sucrose gradients of uridine-\(^3\)H-labeled material from culture supernatants of contaminated cultures produced \(^3\)H peaks at buoyant densities of 1.20 to 1.24 and 1.16 to 1.18 g/ml. Virus titers in culture fluids from Mycoplasma-contaminated cultures were greatly reduced as judged from viral interference tests. The viral RNA was degraded to a low-molecular-weight species when virions were harvested 2 to 24 hr after labeling of Mycoplasma-contaminated cultures.

MATERIALS AND METHODS

Cells and Virus. A line of C3H mouse embryo cells which releases KiMuLV was grown in Earle's Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum, glutamine, sodium bicarbonate, penicillin, streptomycin, and polymyxin (5, 9, 15). Nearly confluent cells were exposed for 24 hr to \(^3\)H-labeled uridine, 10\(\mu\)Ci/ml (specific activity, 33 Ci/mmole; Schwarz/Mann, Orangeburg, N. Y.), or to carrier-free \(^32\)P, 20 \(\mu\)Ci/ml, in 50 ml growth medium (New England Nuclear, Boston, Mass.). Culture fluids were harvested 2 or 24 hr after labeling and purified by centrifugation through discontinuous or linear sucrose gradients (5, 15, 21). Cellular and viral RNA's were analyzed by electrophoresis in 2% acrylamide:0.5% agarose gels as described (5).

Mycoplasma Assays. All cultures were tested weekly. A mixture of growth medium and cell suspension (at least 24 hr in culture) was spread on agar plates made by Hayflick's method (1-3, 12, 18). Plates were inspected under indirect lighting for the presence of "fried-egg" or "tiny" colonies.

Treatment of My\(^+\) Cell Cultures. Several methods for eradicating Mycoplasma contamination of tissue cultures have been recommended (1-3, 18). We found empirically that treatment with gentamicin, 500 \(\mu\)g/ml (Schering Corp. Port Reading, N. J.), for 3 days in Medium 199 (without other antibiotics) followed by gentamicin, 100 \(\mu\)g/ml, for 4...
days eliminated *Mycoplasma* contamination from *My*+ cell cultures.

**Viral Interference Test.** Supernatant fluids from unlabeled cell cultures were harvested 2 or 24 hr after the last medium change when the cells were nearly confluent. The culture fluids were purified by centrifugation through discontinuous or linear sucrose gradients in Tris buffer, pH 7.2. The 40 and 50% sucrose fractions of discontinuous gradients were collected as described and dialyzed overnight at 4°C against Tris buffer, pH 7.2 (5, 21). Fractions with buoyant densities of 1.16 to 1.18 and 1.20 to 1.24 g/ml after centrifugation through 30 to 60% (w/v) sucrose gradients were dialyzed similarly (21). The material was diluted in Tris buffer and immediately used to infect Swiss/3T3 cells on Day 0. The cells had been seeded in 60-mm plastic dishes 2 days earlier at a density of 2.5 × 10^4/dish. The cells were treated 1 day prior to virus infection with DEAE-dextran (25 μg/ml for 1 hr at 36°C) (14). Swiss/3T3 cells were grown in fortified Eagle’s medium and subcultured weekly (16). Medium was changed every 2 days. Duplicate plates were infected with 0.5 ml stock KiMSV on Day 21; control plates were exposed to the same amount of stock KiMSV only. Foci of transformed cells were counted on Day 28 after infection with KiMuLV or on Day 7 after KiMSV infection (16). Interference was expressed as % reduction in the number of foci and calculated as before (6).

**RESULTS**

The viability of *My*+ cells depended on the size of the culture vessel. The cells grew without developing overt cytopathic effects in 60-mm plastic dishes, and saturation densities after an 8-day growth cycle were the same for *My*+ cells and *My*- cells. Cell lysis or other signs of cell damage were likewise absent when the *My*+ cells were propagated in 30-oz prescription bottles. However, the saturation densities of *My*+ cells were lower (by approximately 100,000 cells/bottle) than the saturation densities of *My*- cells. In addition, culture fluids were purified by centrifugation through discontinuous or linear sucrose gradients in Tris buffer, pH 7.2. The 40 and 50% sucrose fractions of discontinuous gradients were collected as described and dialyzed overnight at 4°C against Tris buffer, pH 7.2 (5, 21). Fractions with buoyant densities of 1.16 to 1.18 and 1.20 to 1.24 g/ml after centrifugation through 30 to 60% (w/v) sucrose gradients were dialyzed similarly (21). The material was diluted in Tris buffer and immediately used to infect Swiss/3T3 cells on Day 0. The cells had been seeded in 60-mm plastic dishes 2 days earlier at a density of 2.5 × 10^4/dish. The cells were treated 1 day prior to virus infection with DEAE-dextran (25 μg/ml for 1 hr at 36°C) (14). Swiss/3T3 cells were grown in fortified Eagle’s medium and subcultured weekly (16). Medium was changed every 2 days. Duplicate plates were infected with 0.5 ml stock KiMSV on Day 21; control plates were exposed to the same amount of stock KiMSV only. Foci of transformed cells were counted on Day 28 after infection with KiMuLV or on Day 7 after KiMSV infection (16). Interference was expressed as % reduction in the number of foci and calculated as before (6).

Whole-cell extracts from various *Mycoplasma*-contaminated mammalian cell cultures contain 23 and 16 S RNA's, which are considered characteristic of mycoplasmal and other prokaryotic RNA's (4, 18). We analyzed whole-cell extracts from 32P-labeled *My*+ cells and from uridine-3H-labeled *My*- cells by electrophoresis in separate 2% acrylamide:0.5% agarose gels as described (5). The electrophoretic profiles of whole-cell RNA from *My*+ (—) and *My*- (-----) cells are shown.

Chart 1. Electrophoretic chromatograms of whole-cell RNA extracted from *My*+ cells and *My*- cells. Nearly confluent *My*+ cells were exposed for 24 hr to 3H-labeled uridine, 10 μCi/ml; cultures of *My*- cells were exposed to carrier-free 32P, 50 μCi/ml, in 50 ml growth medium. Whole-cell extracts were prepared and subjected to electrophoresis in separate 2% acrylamide:0.5% agarose gels as described (5). The electrophoretic profiles of whole-cell RNA from *My*+ (—) and *My*- (-----) cells are shown.

Viral yield was compared by determining 3H radioactivity in culture fluids of labeled *My*+ and *My*- cells. Culture fluids were harvested 2 or 24 hr after a 24-hr labeling period, spun through a 5% sucrose barrier, and centrifuged through discontinuous sucrose gradients. This procedure was chosen because most cellular debris is eliminated from the final viral preparation (5). The gradient profiles from 24-hr harvests are shown in Chart 2. 3H radioactivity was distributed in *My*- cell culture fluids as expected (5). Approximately 30% of total radioactivity was recovered from each of the pelleted and 50% sucrose gradient fractions; another 10% of total radioactivity was present in the 40% sucrose gradient fraction. Radioactivity was consistently lower in culture fluids of *My*+ cells than of *My*- cells. The comparatively large 3H peak in the 40% sucrose fraction from *My*+ cells disappeared after the cells had been treated with gentamicin for 9 days (“Materials and Methods”). Agar assays of the previously *My*+ cells for *Mycoplasma* became negative after this treatment, suggesting that the peak was mainly due to *Mycoplasma*. Except for low levels of 3H radioactivity, gradient profiles from 2-hr culture fluids of *My*+ cells and *My*- cells were similar to those shown in Chart 2.
Mycoplasma and Mouse Leukemia Virus Growth

Chart 2. Virus purification by centrifugation through discontinuous sucrose gradients. Nearly confluent C3H mouse embryo cells were exposed for 24 hr to 3H-labeled uridine, 10 μCi/ml. Virus-containing culture fluids were harvested 2 or 24 hr after labeling and centrifuged at 20,000 x g for 20 min. The uridine-3H material was spun at 88,500 x g for 70 min through a 5% sucrose cushion. The pellet was resuspended in 0.2 ml Tris buffer, layered on 5-ml discontinuous sucrose gradients (1 ml 30% sucrose, 2 ml 40% sucrose, and 2 ml 50% sucrose), and centrifuged at 243,000 x g for 2 hr. Peak fractions were diluted with Tris buffer and pelleted in a Spinco Ti 50 rotor at 226,400 x g for 1 hr. Peak areas of the gradients approximating the 50 and 40% sucrose layers as well as the gradient pellet are identified. My* cell culture fluids ( ) are compared with My cell culture fluids ( ).

The 2nd method of virus purification used for these experiments involved a high-speed clarification of culture fluids followed by isopycnic banding in 30 to 60% sucrose gradients. Uridine-3H-labeled KiMuLV from My* cell culture fluids banded at buoyant densities of 1.16 to 1.18 g/ml whether 2- or 24-hr culture fluid harvests were used as virus source (5, 15, 21). Equilibrium centrifugation of My* cell fluids resolved the labeled material into a small 3H peak at buoyant densities of 1.16 to 1.18 g/ml and a broad 3H peak at buoyant densities of 1.20 to 1.24 g/ml (Chart 3). Some gradients from My* cells banded at densities extending from 1.16 to 1.24 g/ml, especially when the culture fluids were harvested 24 hr after labeling. Todaro et al. (20) have shown that labeled mycoplasmas from mammalian cell cultures band at densities 1.20 to 1.24 g/ml. Agar plates of these fractions from My* cell cultures developed characteristic fried-egg colonies.

The low radioactivity recovered from My* cell culture fluids cannot be interpreted as evidence for the suppression of virus growth in these cultures since Mycoplasma contamination is known to interfere with the uptake of thymidine or uridine by cultured cells (7). The yield of KiMuLV from My* cells and My cells was therefore compared by a viral interference test using unlabeled cells as virus source. These assays are based on interference with focus formation by murine sarcoma virus after the cells are first infected with MuLV (13, 16). Table 1 shows the result of viral interference tests with culture fluids sampled 2 hr after labeling. Preinfection of 3T3 cells with undiluted virus material from My* cell cultures reduced focus counts to 16 to 18% of the controls. In contrast, KiMuLV preparations from My* cell culture fluids interfered with focus formation to a significant degree in dilutions up to 10^-4. Differences of 10% or less in focus counts were not considered significant because of the variations in counting foci of transformed cells. The 40% sucrose fractions of the discontinuous gradients as well as buoyant density fractions of 1.20 to 1.24 g/ml caused cytopathic effects in 3T3 cells within 7 to 14 days after infection, and cell-culture fluids from these 3T3 plates were positive for Mycoplasma (12). We concluded that only the 50% sucrose fraction and density fractions of 1.16 to 1.18 g/ml from My* cells contained KiMuLV capable of interfering with focus formation by KiMSV, although virus titers were significantly lower in culture supernatants of My* cells than in My- cells.

For determination of whether Mycoplasma contamination of the virus-producing cell cultures altered the configuration of the viral RNA, culture fluids from My* cells and My- cells were purified by discontinuous sucrose gradient centrifugation. The viral RNA was extracted by treatment with sodium dodecyl sulfate and analyzed by electrophoresis in 2% acrylamide:0.5% agarose gels. Since the RNA of KiMuLV is degraded by aging of the virus in spent growth medium for 12 or 24 hr (5), RNA profiles were examined...
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Table 1
Assays of culture fluids from My* cells and My* cells for viral interference

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>Dilution of viral pellet</th>
<th>Ratios of focus counts*</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>My* cells</td>
<td>10°, 10⁻¹, 10⁻²</td>
<td>3/112 (97)</td>
</tr>
<tr>
<td></td>
<td>10⁻³</td>
<td>14/112 (88)</td>
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<tr>
<td></td>
<td>10⁻⁴</td>
<td>36/112 (69)</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>88/112 (22)</td>
</tr>
<tr>
<td>My* cells</td>
<td>10°</td>
<td>94/112 (16)</td>
</tr>
<tr>
<td></td>
<td>10⁻¹</td>
<td>102/112 (9)</td>
</tr>
<tr>
<td></td>
<td>10⁻², 10⁻³, 10⁻⁴</td>
<td>108/112 (4)</td>
</tr>
</tbody>
</table>

* The average number of KiMSV-induced foci in duplicate dishes of KiMuLV-infected 3T3 cells was divided by the average number of foci in duplicate dishes of 3T3 cells infected with KiMSV only.

DISCUSSION

The present experiments deal with the growth of a MuLV in an established, virus-producing mouse cell line which became contaminated with an unidentified Mycoplasma species. The My+ cells released low levels of uridine-³H-labeled virus into the culture fluids in contrast to My* cells. Isopycnic banding of My+ cell culture fluids in sucrose gradients yielded ³H peaks in the density range (1.20 to 1.24 g/ml) characteristic of labeled Mycoplasma (Charts 2 and 3) (20). The contaminating organisms were probably associated with the 40% sucrose fractions when the culture fluids were purified by centrifugation through discontinuous sucrose gradients (5). This conclusion is based on the repeated
observation that the comparatively large $^3$H peak in the 40% fraction from My$^+$ cell culture fluids disappeared following treatment with gentamicin. Further, the 40% sucrose fraction from My$^+$ cells, but not from My$^-$ cell culture fluids, caused cytopathic effects in 3T3 cells infected with this fraction for the viral interference tests.

Although the viral interference tests indicated significantly reduced virus titer in culture supernatants of My$^+$ cells, several other interpretations must be considered to explain this finding. Mycoplasmas are known to deplete the growth medium of essential metabolites because of their predominantly extracellular growth (3, 18). The poor virus yield from My$^+$ cells may therefore reflect the difficulties in maintaining comparable densities of virus-producing cells in the 2 types of cultures. We attempted to correct for a possible cell loss by using large roller bottles for virus production. Cell loss was minimal under these growth conditions provided the cells were fed every 2 hr. It is also conceivable that contamination of the gradient-purified My$^+$ cell supernatant fluids by Mycoplasma interfered with focus formation in 3T3 cells. Such interference has been reported for chick embryo and human cells which became partially resistant to transformation by Rous sarcoma virus following infection with M. orale or Mycoplasma hominis (11, 17). However, the 3T3 cells used for the present viral interference tests were invariably killed when infected with gradient fractions containing Mycoplasma. Another explanation for the reduced virus titers in culture supernatants of My$^+$ cells is offered by our findings on the properties of the viral RNA. The KiMuLV progeny released into My$^+$ cell culture fluids did not contain the typical high-molecular-weight RNA; instead, the RNA was degraded to low-molecular-weight species (Chart 4). This finding cannot be explained on the basis of thermal degradation since the virions were sampled 2 hr after labeling and since the RNA from KiMuLV of My$^-$ cell culture fluids had properties characteristic of oncornaviruses (5, 8, 19). Whatever the reason for the degraded viral RNA, we are not aware of previous reports on oncogenic or nononcogenic viruses showing configurational changes of the viral genome after Mycoplasma had infected the cell cultures used for their production (3, 18).

It is now widely appreciated that long-term mammalian cell cultures are frequently contaminated by Mycoplasma (3, 18). Our observations emphasize the need for continuous monitoring of MuLV-producing cells for Mycoplasma to avoid misinterpretations about virus structure and replication.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Beem for advice on Mycoplasma isolation, C. Raineri for technical assistance, and J. Berry for typing the manuscript.

REFERENCES

Mouse Leukemia Virus Growth in Mouse Cells Contaminated with *Mycoplasma*

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