Infection of an Established Mouse Bone Marrow Cell Line (JLS-V9) with Rauscher and Moloney Murine Leukemia Viruses

BRUCE S. WRIGHT, PATRICIA A. O'BRIEN, GEORGE P. SHIBLEY, SAMI A. MAYYASI, AND JENNIE C. LASFARGUES

The John L. Smith Memorial for Cancer Research, Chas. Pfizer & Company, Inc., Maywood, New Jersey 07607

SUMMARY

A cell line (JLS-V9) established from the bone marrow of normal weanling BALB/c mice has been shown to be susceptible to infection with either the Rauscher or Moloney murine leukemia viruses. The Rauscher- and Moloney-virus-infected cultures were designated JLS-V10 and JLS-V11, respectively. Both infected cultures were carried for 7 months without loss of infectivity. However, more recent bioassays of Rauscher virus from the JLS-V10 line have indicated a reduction of infectivity for BALB/c mice. Virus multiplication was evaluated both by electron microscopy and bioassays of cell free tissue culture fluids. The viruses were identified by serum neutralization and response of infected mice to the respective viruses, as well as electron microscopy. Cytopathogenic effects of the viruses have not been observed in either of the infected cultures.

INTRODUCTION

Approximately two years ago, successful cultivation of the Rauscher murine leukemia virus in a spleen and thymus cell line from normal BALB/c mice was reported (9). At that time, reference was made to the fact that the control noninfected culture was carrying a latent noninfectious virus which was morphologically indistinguishable from the infectious murine leukemia virus described by Rauscher (5). Although this latent agent does not appear to interfere with the multiplication of the murine virus, it does hinder the interpretation of results from studies involving the control cell line. Therefore, efforts were made to establish new cell lines, free of latent agents and susceptible to infection with the murine leukemia viruses.

This study not only confirms the previous report that the Rauscher virus can be propagated in vitro for prolonged periods, but describes a new cell line which also supports the multiplication of both the Rauscher and the Moloney viruses. To date, replicating noninfectious "C"-type particles have not been detected in this line.

MATERIALS AND METHODS

Medium. Difco's NCTC 109 medium, modified as previously described (9), was used throughout these studies.

Establishment of the Control Mouse Bone Marrow Cell Line—JLS-V9

Bone marrow was collected aseptically from the tibial and femoral bones of the hind legs from two weanling BALB/c mice. After removal of the knee joints, the marrow was flushed from the bones by forcing cold Gey's balance salt solution through the marrow cavity. The bone fragments were washed several times and the washings pooled in a sterile centrifuge tube. The cell suspension stood for five minutes in an ice bath and the supernatant, containing the more uniform cellular suspension, was transferred to another tube and centrifuged at 500 X g for five minutes. The cell pellet was resuspended in modified 109 growth medium without sodium bicarbonate. Approximately 2 X 10^7 cells were seeded into T-flasks. Due to acidity, the medium was changed two days later to a modified 109 growth medium containing 0.22 percent sodium bicarbonate. The cultures were fed every two days and maintained this way for approximately one month without transfer. When the cell sheets were confluent, the cultures were trypsinized for the first time with 0.05 percent trypsin in Puck's saline D solution (T. Puck, personal communication). The cells were centrifuged and resuspended in the pooled supernatant growth medium from the original cultures (conditioned medium) and seeded into another T-flask. The culture was refed when necessary and three months had elapsed before a second transfer was made. The third passage was made within a month while the fourth transfer was prepared in nine days. At the fifth passage, the cells were subdivided into two flasks for the first time. Thereafter, the cultures were transferred routinely once a week. Cells from the 24th transfer of the control mouse bone marrow cell line, designated JLS-V9, were frozen in a Linde liquid nitrogen freezer.

Rauscher-Virus-Infected Mouse Bone Marrow Cell Line (JLS-V10)

The 24th passage of the JLS-V9 control mouse bone marrow cell line was removed from the nitrogen freezer after a year's storage and passed 18 times before infecting with the Rauscher virus. The virus source used to establish the infection was bone marrow cells removed from a mouse 24 days after infection with standard Rauscher mouse plasma virus.

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Bone marrow cells from the infected mouse were collected in Hank's balanced salt solution without sodium bicarbonate. The procedure was the same as that described for the noninfected cells. The pellet of infected cells was resuspended in a small volume (2 ml) of modified growth medium. One and a half ml of this cell suspension were added directly to a monolayer (18th passage) of the normal mouse bone marrow (JLS-V9) culture. After adsorption for one hour at 37°C, growth medium was added to the culture. After infection, this 18th transfer was designated JLS-V10. This culture is currently in the 117th passage since infection while the control JLS-V9 has been passed 135 times since the culture was taken from the freezer.

**Moloney-Virus-Infected Mouse Bone Marrow Cell Line (JLS-V11)**

The Moloney virus source used to infect the control JLS-V9 cell line, was an infected mesenteric lymph node taken from an infected mouse 42 days after inoculation with Moloney mouse plasma virus. The node was washed and cut into small pieces by the cross blade technic, and the rest of the procedure was the same as described for the Rauscher virus. The lymphocytes were added to the 13th passage of the control mouse bone marrow (JLS-V9) cell line. The next day, it was found that the mixed cell population had peeled from the glass. Therefore, 4–5 ml of the mixed cell suspension were added to a freshly prepared culture of JLS-V9. This 14th passage became JLS-V11 or the Moloney-virus-infected mouse cell line. This culture is currently in its 107th passage since infection.

**Mouse Tests.** Weanling BALB/c mice were inoculated intraperitoneally with 0.1 ml of unprocessed or concentrated cell free culture material from the JLS-V10 cell line. Newborn BALB/c mice were similarly inoculated with cell free preparations from the JLS-V11 cell line. Each sample of the Moloney virus was inoculated into two litters of 48-hour-old mice, while ten 3- to 4-week-old weanlings were used for each Rauscher virus sample. The disease syndromes were the same as those described by Rauscher (5) and Moloney (4). Mice inoculated with the Moloney virus as well as those surviving the early erythrocytopenic phase of the Rauscher virus disease were autopsied four months after inoculation.

### TABLE 1

**Bioassay Data of the Rauscher and Moloney Murine Leukemia Viruses from Infected Mouse Bone Marrow Cell Lines**

<table>
<thead>
<tr>
<th>Tissue culture passage after infection</th>
<th>Days in tissue culture since infection</th>
<th>Assay in vivo</th>
<th>Leukemic/Total</th>
<th>Dead/Total</th>
<th>Fm (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
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<td>Rauscher-virus-infected mouse bone marrow JLS-V10</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>32</td>
<td>6/7</td>
<td>86</td>
<td>5/7</td>
<td>71</td>
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<tr>
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<td>7/8</td>
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<td>90</td>
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<td></td>
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<tr>
<td>3</td>
<td>15</td>
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<td>88</td>
<td>5/8</td>
<td>62</td>
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</tr>
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<td>100</td>
<td>5/9</td>
<td>56</td>
</tr>
</tbody>
</table>

* Observation periods, four months postinoculation.
EXPERIMENTS AND RESULTS

Biologic Tests of Cell Free JLS-V10 and VII Tissue Culture Fluids. Periodically, during the subculturing of infected cells from JLS-V10 and VII, samples were collected and centrifuged to remove cells and cellular debris before animal inoculation. The first collection of tissue culture fluid from JLS-V10 was made after the infected cells had been transferred nine times (Table 1). The induction of leukemia by cell free culture fluids, collected from the 32nd to the 436th day after the cell line was infected, is shown in Table 1. At the time of sacrifice, the mean times to palpation and death for 50 percent of the recipients were calculated. These mean values were estimated by the graphic rankit method (8).

Tissue culture fluid collected from the JLS-V10-infected cells 209 days after infection was as active as that taken after 32 days. Although the mean times to palpation and death were slightly increased, the incidence of leukemia remained high throughout this 7-month period. The JLS-V10 culture maintained a high infectivity through the 60th passage. However, by the 75th transfer, the infectivity had fallen to zero.

The infectivities of concentrated JLS-V10 tissue culture fluids from the 26th, 51st, and 120th passages are also shown in Table 1. Virus activity was not observed with the last sample even though it was five times more concentrated than the first two preparations.

Infectious Moloney virus was found to be present in unprocessed cell free tissue culture fluids collected from the third through the fifty-fifth passages of the JLS-V11 cell line. The cell passage history and the number of days the infected culture has been carried in vitro since infection, are also shown in Table 1. As with the Rauscher-virus-infected mice, the mean times to palpation and death for 50 percent of the recipients were calculated after an observation period of 120 days.

As shown in Table 1, the incidence of leukemia caused by fluid collected from virus-infected cells 242 days after infection was as high as that tested within 15 days. From this incidence of leukemia, it is obvious that virus multiplication occurred continuously throughout the 7-month test period, even though the infected cells had been subcultured 55 times. Although the mean time to palpation was similar for the Rauscher- or Moloney-virus-infected mice, the significant difference is seen in the mean time to death for the respective virus infections. In general, the averaged latent period for death of the JLS-V11-infected mice was 3.3 months, while the same period for the JLS-V10-inoculated mice was 1.5 months. These figures agree favorably with those reported by Moloney (4) and Rauscher (5) for extracts of leukemic tissues. The bioassay response to a 50X concentrate of cell free tissue culture fluid from the 40th passage of JLS-V11 was similar to the unprocessed tissue culture virus.

Serum Neutralization Tests. Immune rabbit sera were prepared against the respective tissue culture viruses. Serum neutralization tests were performed as previously described (9) using these antisera and the respective mouse plasma viruses. Both plasma viruses were shown previously to have an ID₅₀/ml of 10⁻⁴, as calculated by the method of Reed and Muench (6). Normal preimmunization sera from the same rabbits served as the controls for the two tests.

The results of this study (Table 2) indicate a highly significant neutralization of the mouse plasma viruses with rabbit antisera to the respective tissue culture viruses. These results and others identified the tissue culture viruses as the specific murine leukemia agents.

TABLE 2
Serum Neutralization Tests with Normal, Rauscher (JLS-V10), and Moloney (JLS-V11) Immune Rabbit Sera against the Homologous Murine Leukemia Viruses*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Rauscher plasma virus mixed with:</th>
<th>Percent positive log dilution of virus</th>
<th>Average day to palpation</th>
<th>Average day to death</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NRS</td>
<td>100 100 89 14</td>
<td>18 23 47 119</td>
<td>29 43 50 77</td>
</tr>
<tr>
<td></td>
<td>IRS</td>
<td>0 0 0 0 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Moloney plasma virus mixed with:</td>
<td>ND 83 92 18</td>
<td>ND 52 55 75</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>ND</td>
<td>ND 0 0 0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>IRS</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

NRS ID₅₀/ml = 10⁻⁴
IRS ID₅₀/ml = <10⁻³
NI = >2.5 or >320

NRS ID₅₀/ml = 10⁻⁷₈
IRS ID₅₀/ml = <10⁻⁴₀
NI = >2.46 or >280

* ND, Not done; NI, neutralization index; NRS, normal rabbit serum; IRS, immune rabbit serum.

² Observation periods, four months postinoculation.

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sections from the two virus-infected cultures has revealed the characteristic C-type virus particles, usually with an electron-dense nucleoid (Figs. 1, 3). In general, the virus particles were found in the extracellular spaces as well as lining the periphery of the infected cell. Typical budding of the virus particles (Figs. 2, 4) was seen in both the Rauscher- and Moloney-virus-infected cells. Like the mouse plasma viruses, the Rauscher and Moloney tissue culture viruses were morphologically indistinguishable from one another.

DISCUSSION

At the time of this report, the two virus-infected cell lines have been carried for approximately 7 months. Since the initial detection of virus, these chronically infected cultures have constantly released virus into the tissue culture medium.

During this study, both virus-infected and control cells were repeatedly tested for other contaminants and found to be free of pleuropneumonia-like organisms, etromelia, polyoma, mouse hepatitis virus, and the lactic dehydrogenase agent.

Although virus is constantly being collected from the JLS-V10 and V11 cell lines, the conditions under which these cultures were infected leave the possibility that the infected cells from the inoculum were perpetuated in culture, analogous to a feeder-layer system, without transferring the virus to the normal cell. However, except for budding virus from the infected cultures, the three cell lines were morphologically indistinguishable when thin sections of cell pellets were examined by electron microscopy. In addition, the control V9 cells were susceptible to infection with cell-free mouse plasma preparations of both viruses. Regardless of whether a mixture of cell types exists or not, the systems have proven to be effective for the propagation of these murine leukemia viruses. It is interesting to note that on at least two different occasions, the Rauscher virus from V5 (10) and V10 has lost most of its infectivity for weanling BALB/c mice after long-term in vitro propagation, while no such indication has been seen, at least at the time of this writing, with the Moloney virus. Both Manaker’s MT-77 culture (3) and the JLS-V11 are still producing infectious virus after prolonged culture.

The infectivity of Rauscher virus from early passages of the JLS-V10 was similar to that reported for the same virus from an established mouse spleen and thymus (JLS-V5) culture (9). However, more recent bioassay data of the V10 virus have indicated a marked drop in the infectivity for weanling BALB/c mice following intraperitoneal inoculation with clarified cell free tissue culture fluids. Similar low leukemogenic strains of Rauscher virus have been reported (7). Although the leukemogenic activity of the V10 virus has shown a marked decrease with continuous subpassage, the cell line has continued to produce high concentrations of the virus. This situation is similar to that observed with the V5 virus and suggested the possibility of using the live V10 virus as an immunizing agent against the lethal effects of Rauscher mouse plasma virus, as was done with the V5 virus (1, 2, 10). However, preliminary results of such experiments indicate that the V10 virus will not protect mice against a lethal challenge of the mouse plasma virus. These results seem to indicate that the V10 virus is less antigenic than the V5 and mouse plasma viruses. The possibilities of either antigenic similarities or differences are currently under investigation.

ACKNOWLEDGMENTS

We wish to thank Drs. Frank J. Rauscher and John B. Moloney, Department of Viral Oncology, National Cancer Institute, for valuable advice, encouragement, and review of the manuscript. Sections of the Moloney- and Rauscher-virus-infected cells were examined by Mr. Richard Stephens of the Viral Oncology Department, Chas. Pfizer & Co., Inc.

REFERENCES

Fig. 1. Osmium-fixed section of Rauscher-virus-infected JLS-V10 cells. Note the characteristic C-type particles in the extracellular spaces. $\times 42,000$.

Fig. 2. Typical Rauscher virus particle budding from the surface of JLS-10 cells as seen in osmium-fixed sections. $\times 129,000$. 
Bone Marrow Infection with Leukemia Virus

Fig. 3. Characteristic C-type particles in the extracellular spaces of Moloney-virus-infected JLS-V11 cells. Most of the virus particles possess electron-dense nucleoids. × 42,000.

Fig. 4. Osmium-fixed section of JLS-V11 cells illustrating a late stage of Moloney virus maturation from the cell surface. Note the cellular membrane surrounding the viral nucleoid. × 100,000.
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