Recovery of Rauscher Leukemia Virus from Large Volumes of Seeded Cow's Milk and from Infected Murine Spleens

EDWARD P. LARKIN AND RAY M. DUTCHER

Section of Viral Oncology, Department of Clinical Medicine,
School of Veterinary Medicine, Kennett Square, Pennsylvania 19348

Received for publication 3 April 1970

Rauscher murine leukemia virus was used as an indicator agent to develop a methodology for the extraction and concentration of a theoretical leukemia virus from bovine milk and tissues. The indicator virus was seeded into cow's milk or was recovered from infected murine spleens. The tissue homogenates and the defatted milk were processed in a B-XVI rotor of a Spinco L-4 ultracentrifuge at a flow rate of 3 liters/hr. The efficiency of Rauscher virus recovery was greatest when the rotor was used without a gradient. A loss of between 0.6 and 0.7 log of total infectious virus, as determined by the spleen assay method, resulted when the seeded milk and murine spleens were processed. The procedures developed are presently being used in transmission experiments in an attempt to induce leukemia in the bovine.

The finding of virus-like particles in milk from leukemic cows (7, 8) and the development of methods for their recovery and partial purification have been reported (7, 15, 16). Similar particles have been observed in lymph node and mammary gland biopsies as well as in tissue cultures from cattle with lymphosarcoma (8, 20, 22, 30). More recently, budding virus particles have been seen in mixed cell cultures (3, 17) and in phytohemagglutinin-stimulated short-term lymphocyte suspension cultures from leukemic cattle (18). The buoyant density and the size and morphology (7, 8) of the particles in milk are similar to the values reported for murine (23) and feline (25) leukemia and sarcoma viruses.

Transmission experiments have demonstrated that murine leukemia is transmissible to newborn mice by inoculation of tissue extracts, filtrates, and plasma (9, 12, 13, 19, 24). Vertical transmission has also been demonstrated by foster nursing studies (4, 5, 13, 19, 20), and virus has been shown to be present in the milk of mice known to have a high incidence of leukemia (6). The transmission of feline leukemia and sarcoma in kittens by the inoculation of tissue extracts and filtrates containing virus (25) has also been confirmed. Attempts to similarly transmit leukemia in cattle by use of viable cells and tissue extracts from leukemic donors have thus far been unsuccessful (11, 14, 26-28). Where responses have been reported, lymphocytosis was often the sole criterion of infection, controls were lacking, or too much time elapsed before cases occurred.

Transmission studies in laboratory animals and cell culture systems with both crude and purified bovine extracts have been unsuccessful. Various methods of concentration and purification, including differential, density gradient, and zonal ultracentrifugation (1, 10, 29) and zonal electrophoresis, have been utilized to concentrate and purify a possible leukemia virus in the bovine system. Electron microscopic examination, which still remains the sole means of detecting virus in the bovine, has revealed the presence of virus-like particles, resembling the "C" type murine leukemia viruses, in various concentrations in all of these bovine preparations.

Therefore, it was essential that a procedure be devised for the extraction of virus-like particles in sufficient quantity from bovine milk and tissues for transmission studies in newborn calves. Although the concentration of virus in milk and tissues of affected cattle is thought to be low, large quantities of raw materials were available. To establish a procedure for the recovery of a bovine leukemia virus and to determine the effectiveness of procedures already developed, an indicator agent, murine leukemia virus (Rauscher), was added to cow's milk or was extracted from virus-containing mouse spleens.

This report describes the effectiveness of the concentration procedures employed in terms of
the quantitative recovery of the indicator agent as measured by its biological activity in mice (2).

MATERIALS AND METHODS

Milk. The milk was processed through a DeLaval model 100 cream separator at a minimum temperature of 31 C.

Indicator virus. Rauscher leukemia virus recovered from the plasma of infected mice was obtained from John Moloney, National Cancer Institute, Bethesda, Md. The Rauscher virus-infected spleens were provided by Earlring Jensen of the John L. Smith Memorial for Cancer Research, Maywood, N.J.

Tissue extracts. Tissues were cut into sections, approximately 2 to 3 cm², and processed immediately or sealed in plastic bags and stored in a Virtis freezer at —87 C until used. The frozen tissues in plastic bags were rapidly thawed by immersion in warm water (37 C) and immediately ground in a General commercial meat grinder (model D). The resulting finely ground tissue was suspended in 2 to 3 volumes of 0.15 M citrate buffer and blended in a Waring Blender (model CB-5), having a 1-gal capacity (ca. 3.8 liters), for 30 sec by use of a series of 10 3-sec bursts.

Centrifugation. Low-speed centrifugation was carried out in a Sorvall RC-2B centrifuge with a GSA rotor. For high-speed centrifugation, an International B-60 ultracentrifuge and an A-170 rotor were employed. Continuous-flow centrifugation was accomplished with a Beckman-Spinco model L-4 ultracentrifuge. A B-XVI rotor was used with and without a 400-ml sucrose cushion. When sucrose was used, a natural gradient was created during the centrifugation procedure. Fractions were removed by pumping from the edge with a 50% sucrose solution.

Sterilization. All metallic parts that came in contact with the sample were autoclaved, with the exception of the centrifuge rotors. The rotors were assembled, with all plastic tubing connected, and sterilized in place by using 12% ethylene oxide (Oxyfume 12; Linde) at 10 lb of pressure for a minimum of 12 hr. The ethylene oxide was removed by passing dry nitrogen at 10 lb of pressure through the system, followed by the flushing of 5 liters of sterile sodium citrate buffer through the rotor. The centrifuge tubes were sterilized by either autoclave or by ultraviolet light. All equipment and glassware contaminated during the processing of the sample were disinfected by soaking in a formaldehyde-detergent bath.

Other. Twenty-liter sterile stainless-steel pressure vessels (Millipore Corp., Bedford, Mass.) were used to store the sample for processing through a B-XVI rotor. The vessels were immersed in an ice bath, and the sample was maintained at 5 C.

Mouse spleen assay. Portions of serial dilutions of samples (0.1 ml) were inoculated intraperitoneally into 4-week-old BALB/cCr mice. After 21 days, the mice were killed, and spleen weights were recorded to the nearest milligram. Spleens of more than 180 g were considered positive. Fifteen mice were used for each dilution group. Spleen assays were performed by Gerald Spahn (Microbiological Associates, Inc.).

RESULTS

Because of the variation in the volume at different sampling points of milk and tissue extracts, it was necessary to compare the total amount of infectious virus present at each step in the process. Infectivity, as determined by the mouse-spleen assay method, was reported as the ED₅₀ per milliliter of the sample. The volumes of the fluids were converted to logarithms and added to the logarithms of the infectivity titers, the result being the total amount of infectious units at each stage of the process.

During the operation of the B-XVI rotor, an approximate linear gradient was created as the sucrose diffused into the covering citrate buffer. In the initial run, a flow rate of 6 liters/hr was used to process the milk seeded with Rauscher virus. After the flow-through of the sample was completed, the rotor was maintained at operating speed (38,000 rev/min) for an additional 1 hr to sharpen the zones in the gradient. The results of this experiment are shown in Table 1. An initial reduction of virus infectivity occurred when the inoculum was mixed with the raw cow's milk. Whether this loss was due to a sampling error or to a virucidal effect of the milk was not determined. The 1.14 to 1.17 g/cm³ sucrose gradient fraction showed an additional loss of virus. After dialysis to remove the sucrose and after filtration, the original virus concentration experienced a four-log reduction of infectivity.

In an attempt to increase the yield of virus, two additional Rauscher virus-seeded milk samples were processed as follows; the first

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flow rate (6 liter/hr)</th>
<th>Flow rate (3 liter/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus pool</td>
<td>7.03 (270 ml)</td>
<td>7.24 (100 ml)</td>
</tr>
<tr>
<td>Milk + virus pool</td>
<td>5.86 (8,670 ml)</td>
<td>5.18 (4,100 ml)</td>
</tr>
<tr>
<td>Virus pool + PBS³</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cream</td>
<td>3.97</td>
<td>6.86</td>
</tr>
<tr>
<td>Skim</td>
<td>ND</td>
<td>5.23</td>
</tr>
<tr>
<td>Citrated skim</td>
<td>5.69</td>
<td>6.00</td>
</tr>
<tr>
<td>1.14 to 1.17 Gradient fraction</td>
<td>4.23 (85 ml)</td>
<td>5.91 (210 ml)</td>
</tr>
<tr>
<td>Postdialysis</td>
<td>4.35</td>
<td>5.42</td>
</tr>
<tr>
<td>Postfiltration</td>
<td>3.26</td>
<td>4.80</td>
</tr>
</tbody>
</table>

* Spleen assay.
³ Phosphate-buffered saline.
⁷ No determinations.
observed the same procedure as described earlier with the exception of a reduced flow rate of 3 liters/hr. The second sample was processed at 3 liters/hr without a sucrose gradient, and the pellet was removed with a sterile spatula from the wall of the rotor. Results of these experiments are described in Tables 1 and 2.

With the reduced flow rate, the loss of infectious units in the gradient fraction was reduced to 1.33 logs, as compared to 2.8 logs for the 6-liter/hr flow rate. When the sample was pelleted in the absence of a gradient at a flow rate of 3 liters/hr, the loss was reduced further to 0.68 log.

In an attempt to develop procedures for extracting virus from tissues, 870 g of Rauscher-infected murine spleens was processed by the procedures outlined in Fig. 2. The results are shown in Table 3. The 8,700 ml of the homogenate was reduced 44-fold with a loss of 0.6 log of infectious units. The total initial infectivity was reduced from 7.84 to 7.21 logs.

**TABLE 2. Recovery of infectious virus from bovine milk seeded with Rauscher leukemia virus by using the B-XVI rotor without a gradient**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Logarithm of the total infectious virus in the sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus pool</td>
<td>6.95 (255 ml)</td>
</tr>
<tr>
<td>Milk + virus pool</td>
<td>6.09 (8,035 ml)</td>
</tr>
<tr>
<td>Virus pool + PBS†</td>
<td>6.95</td>
</tr>
<tr>
<td>Resuspend pellet recovered from rotor wall…</td>
<td>6.27 (180 ml)</td>
</tr>
<tr>
<td>Postcentrifugation (low-speed)</td>
<td>5.61</td>
</tr>
</tbody>
</table>

† Flow rate, 3 liters/hr.
‡ Spleen assay.
§ Phosphate-buffered saline.

**TABLE 3. Recovery of infectious virus from murine spleens containing Rauscher leukemia virus by using the B-XVI rotor without a gradient**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Logarithm of the total infectious virus present in the sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen homogenate</td>
<td>7.84 (8,700 ml)</td>
</tr>
<tr>
<td>Resuspend pellet recovered from rotor wall…</td>
<td>7.21 (200 ml)</td>
</tr>
<tr>
<td>Postcentrifugation (Sorvall RC-2B, 3,000 rev/min, GSA rotor, 10 min)</td>
<td>7.12</td>
</tr>
<tr>
<td>Postfreezing (−87°C)</td>
<td>6.53</td>
</tr>
</tbody>
</table>

† Flow rate, 3 liters/hr.
‡ Spleen assay.

**DISCUSSION**

Because of the difficulty and uncertainty involved in attempting to monitor extraction, concentration, and purification processes by electron microscopy, an indicator organism, Rauscher murine leukemia virus, was used to monitor the methodology and was seeded into the milk and recovered or was extracted from infected mouse spleens.

In the initial experiment with a flow rate of 6 liters/hr, infectivity studies showed that there was a loss of 2.8 logs of the seeded Rauscher leukemia virus when the 1.14 to 1.17 g/cm² fraction was assayed. Reduction of the flow rate to 3 liters/hr increased recovery, but there was still a loss of 1.33 logs of infectious units. Losses of such magnitude were unacceptable, especially since it was suspected that a bovine leukemia virus, if present at all in the bovine preparations, would be present in low titer.

In an attempt to improve the virus recovery rate, the use of a gradient was eliminated, and the virus was pelleted directly onto the wall of the rotor. The increase in the recovery of the seeded virus by this change in methodology was demonstrated by a further reduction of the infectivity titer from 6.95 to 6.27 logs. A loss of 0.7 log of the initial seeded virus was considered acceptable, considering the volume of milk processed, the loss of fluids by mechanical operation of the system, and the limitations of the assay procedure. Therefore, it was determined that the methodology outlined in Fig. 1 would be utilized in an attempt to extract a possible bovine leukemia virus from milk obtained from cows diagnosed as positive cases of lymphosarcoma.

Tissues recovered from a cow presented unusual technical problems because of the volumes of raw material available. Spleens from infected animals weighed up to 4,000 g, whereas tumors from...
advanced cases varied in weight from a few grams to well over 5,000 g. To process tissues of such magnitude within a relatively short period of time, the technique for the extraction of murine leukemia virus from tissue as advocated by Moloney (21) was modified and scaled up to process kilogram amounts of material (Fig. 2).

In an attempt to determine the effectiveness of such large-scale tissue extraction procedures, murine spleens were processed by the outlined procedures, and the resulting resuspended pellet was titrated in mice. The loss of the indicator virus was 0.6 log which was considered to be satisfactory considering the magnitude of tissue extracted, the mechanical loss of homogenate in the system, and the limitation of the spleen assay method.

A series of experiments was run to determine the effect of dialysis, filtration, and low-speed clarification of the resuspended pellet on recovery of the indicator virus. Each of these procedures resulted in considerable reduction in virus titer. Therefore, it was determined to use the crude resuspended pellet as inoculum for the transmission studies.

In the event that a bovine leukemia virus might be present in milk and tissues of cattle affected with the disease, would it have properties similar to a murine leukemia virus, such as the Rauscher type used in these studies? The results of these experiments can only tell us of the recoverability of Rauscher virus in terms of the concentrations introduced and can only suggest that similar results might be obtained in recovering a bovine virus, should it have similar properties.

With these limitations in mind, procedures have been developed as outlined in Fig. 1 and 2 for the extraction and concentration of a theoretical 14C-type leukemia virus from cattle diagnosed as positive for lymphosarcoma. Transmission studies have been initiated utilizing these procedures in which extracts from lymph node, spleen, and milk are being used to inoculate newborn calves in an attempt to induce leukemia in these animals.

ACKNOWLEDGMENTS

The authors thank James Elliott for his excellent technical assistance and Louise Wiggins for assistance in the preparation of this manuscript.

This study was supported by Public Health Service contracts PH 43-65-1013 and PH 43-67-697 from the Special Virus Cancer Program, National Cancer Institute, and a Scholar Award from the Leukemia Society of America, Inc. Spleen assays were performed by Gerald Spahn under contract PH 43-67-697.

LITERATURE CITED


