In Vitro Transmission and Propagation of the Bovine Leukemia Virus in Monolayer Cell Cultures

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SUMMARY

This study demonstrates that the bovine leukemia virus (BLV) can infect in vitro cells of human, simian, bovine, canine, caprine, ovine, and bat origin. Cultures of these cells, cocultivated with BLV-infected cells or inoculated with cell-free BLV preparations, continuously showed the presence of cells with the internal BLV antigen as well as BLV-induced syncytia. Virus replication was abundant and increased with passage in bat lung cells and was moderate but constant in fetal canine thymus cells. The amounts of virus released by the simian DBS-FRhL-1 and caprine S-743 cultures were low to moderate during the first 4 to 8 weeks and decreased thereafter. In the infected fetal lamb spleen cell cultures, virus production was low and declined further with passage. Bovine embryonic spleen and human diploid embryonic lung WI-38 cell cultures produced very small amounts of virus only during the first two passages after inoculation despite the fact that they remained infected, as determined by the continuous presence of cell BLV antigen and syncytia. Morphologically and antigenically, the virus particles released by the monolayer cell cultures were indistinguishable from those found in short-term and long-term cultures of BLV-infected bovine lymphoid cells. Repeated electron microscopic examinations and serological tests showed that all the BLV-infected cultures, including those from which the infecting inocula were obtained, were free of the foamy-like bovine syncytial virus, parainfluenza 3 virus, infectious bovine rhinotracheitis virus, bovine viral diarrhea virus, and the maedi-like bovine R-29 virus.

INTRODUCTION

A virus designated BLV and with morphological properties similar to those of the C-type leukemia viruses of other species was detected originally in short-term and long-term cultures of lymphoid cells from leukemic cattle (11, 14, 25, 28). Confirming preliminary evidence reported by Olson et al. (26), studies in this laboratory and by Van der Maaten and Miller (33) have shown that BLV is leukemogenic in sheep. The etiological role of BLV in bovine leukemia has been supported by extensive seroepidemiological studies in carefully characterized herds (9, 10, 12, 13). Immunological analyses using IFA, IFA absorption, and immunodiffusion techniques failed to reveal antigenic relationships between BLV and other oncornaviruses (8, 23, 24). These findings have been confirmed recently by Ressang et al. (27) and by Gilden et al. (16). BLV has also been shown to be antigenically different from the foamy-like BSV, P13, IBR, BVDV, and the maedi-like R-29 viruses (6, 7, 23, 24).

A detailed characterization of BLV has been hampered by the difficulties in obtaining adequately purified virus from the lymphoid cultures. Indeed, in these cultures BLV is produced in rather limited quantities and in association with large amounts of cellular debris. Efforts have been made to propagate BLV in monolayer cell cultures which, based on experience with other oncornaviruses, are expected to release the virus without significant amounts of cellular debris. Recently, Van der Maaten et al. (34) reported on the limited replication of BLV in cell cultures of FLS cells cocultivated with lymphoid cells from BLV-infected cattle. Ressang et al. (27) observed C-type virus-like particles antigenically related to BLV in cell cultures established by explantation of lymph nodes from sheep inoculated with blood from leukemic cattle as well as in cultures of fetal bovine cells cocultivated with peripheral mononuclear cells from a leukemic cow.

Studies in this laboratory have demonstrated that BLV infects and induces syncytia formation in cell cultures of bovine, human, simian, canine, ovine, caprine, and bat origin. Virus replication was noted in some of the infected cultures (6, 17). This report is concerned with a more detailed study of the ability of BLV to infect and propagate in these cultures.

MATERIALS AND METHODS

Cells and Cell Cultures. BLV-infected BC cells were collected from a cow (AJ-147) with persistent lymphocytosis in multiple-case Study Herd BF (9), using procedures previously described (28). Following in vitro cultures for 48 to 72
hr, a large proportion of the AJ-147 BC cells showed BLV particles, as determined by electron microscopy, and the internal BLV antigen, detected by the IFA technique (8–10). Repeated electron microscopic examination and IFA testing with well-characterized reference sera (6) showed that the AJ-147 BC cells were not infected with BSV, PI, IBR, BVDV, or R-29 virus.

A BLV-producing monolayer cell culture, designated FLS·NI-1228, was obtained through the courtesy of Dr. M. J. Van der Maaten (National Animal Disease Center, Ames, Iowa) at passage 22. This culture was established by cocultivating FLS cells with BLV-infected bovine lymphoid cells (34). Uninfected FLS cells were also supplied by Dr. Van der Maaten.

Cell line NBC-13 was established from peripheral lymphocytes of a cow with leukemia and is routinely maintained as a suspension (spinner) culture. Periodic tests have shown that this cell line is free of BSV, PI, IBR, BVDV, and R-29 virus. Details on the derivation, maintenance, and characteristics of cell line NBC-13 have been reported previously (10, 14, 18).

A BSV-infected cell culture, designated BS-2, was derived from a bovine fibrosarcoma. This culture released characteristic BSV particles, reacts strongly in IFA tests with reference BSV serum, and is negative for both BLV particles and BLV antigen(s) (7).

A summary of the origin of these cell cultures as well as those used as hosts in the in vitro transmission experiments with BLV is given in Table 1.

All monolayer cultures were grown in 75-sq cm Falcon tissue culture flasks using 25 ml of Eagle’s minimal essential medium supplemented with 10 to 20% fetal calf serum inactivated (56° g for 30 min), penicillin (100 units/ml), and streptomycin (100 μg/ml). Upon confluence the cells were dispersed with a mixture of 0.05% trypsin and 0.06% EDTA and subcultured. The cultures were transferred twice a week with the exception of the FLS·NI-1228 and FLS cells, which were passed once a week owing to their slower growth rate.

The AJ-147 BC, TbbLu, and BESP cultures were tested for Mycoplasma contamination by Dr. E. M. Levine (The Wistar Institute, Philadelphia, Pa.) using the uridine phosphorylase and/or the agar plate assay (21), and they were found to be negative.

Infection Procedures. Two procedures were used to infect the monolayer cell cultures: (a) cocultivation with BLV-releasing FLS·NI-1228 or AJ-147 BC cells, in which a 75-sq cm Falcon flask containing a semiconfluent monolayer of the host cells was inoculated with approximately 5 to 10 × 10^6 FLS·NI-1228 cells or 50 × 10^6 AJ-147 BC cells. The mixed culture was incubated at 37° for 48 hr after which the unattached cells were removed by aspiration. The monolayer was washed with Hanks’ balanced salt solution, and then 25 ml of complete fresh medium were added. The monolayer cultures were subcultured every 4 to 7 days; (b) infection with cell-free virus preparations, in which CFF were obtained by ultracentrifugation (100,000 × g, 90 min) of 500 to 750 ml of precleared (10,000 × g, 15 min) supernatant fluids from the BLV-releasing cultures. The pellets were resuspended in 5 ml of Eagle’s minimal essential medium with 10% fetal calf serum inactivated (56° g for 30 min) and filtered through a 0.45-μm Nalgene filter. CFF were prepared by filtering preclarified supernatant fluid from the BLV-releasing cultures through a 0.45-μm Nalgene filter. Semiconfluent monolayer cell cultures were pretreated with 20 ml of complete medium containing DEAE-dextran, 25 μg/ml, for 30 min and then inoculated with either 5 ml of CFF or 10 to 15 ml of CFF. After incubation for 2 hr at 37°, 15 to 20 ml of complete medium were added and the cultures were reincubated. Upon confluence, the monolayers were subcultured and handled in the same manner as those inoculated with BLV-releasing cells.

Cytopathological Examinations. Monolayer cell cultures were examined for cytological alterations at every other passage. Cells were seeded in either 25-sq cm Falcon flasks or in Leighton tubes containing coverslips. Upon confluence, the cells were washed with phosphate-buffered saline, containing NaCl (8.5 g/liter), Na_2HPO_4 · 7H_2O (1.64 g/liter), and KHPO_4 (0.54 g/liter) (pH 7.2); fixed with methanol; and stained with Giemsa (6).

RDPD Assay. The RDPD of BLV was assayed applying the method of Todaro et al. (30) with modifications described in Table 1.

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Origin</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A204^</td>
<td>Human rhabdomyosarcoma</td>
<td>2, 15</td>
</tr>
<tr>
<td>DBS-FRhL1^</td>
<td>Rhesus monkey lung</td>
<td>30, 35</td>
</tr>
<tr>
<td>FC1-Th^</td>
<td>Canine thymus (NBRL)</td>
<td>2</td>
</tr>
<tr>
<td>S-743^</td>
<td>Caprine ovary</td>
<td></td>
</tr>
<tr>
<td>TbbLu^</td>
<td>Bovine spleen</td>
<td>2, 30</td>
</tr>
<tr>
<td>FLS^</td>
<td>Bovine embryo spleen</td>
<td>34</td>
</tr>
<tr>
<td>BESP</td>
<td>Human lung</td>
<td>6</td>
</tr>
<tr>
<td>Wi-38^</td>
<td>Chimpanzee spleen (NBRL)</td>
<td>19</td>
</tr>
</tbody>
</table>

| Infected     |        |      |
| AJ-147^      | Bovine BC lymphocytes from a cow (AJ-147) with persistent lymphocytosis | 6 |
| BLV-bat/ / / | TbbLu cells experimentally infected with BLV | |
| BLV-bat/ / / | TbbLu cells experimentally infected with BLV | |
| BS-2^        | Bovine fibrosarcoma; cell culture releasing BSV | 7 |
| FLS·NI-1228^ | FLS cells experimentally infected with BLV | 34 |
| NBC-13^      | Thoracic duct lymphocytes from a leukemic cow; continuous lymphoid cell culture infected with BLV | 10, 14, 18 |

* Supplied by Dr. M. Lieber (Meloy Laboratories, Springfield, Va.).
* Supplied by Dr. J. M. Van der Maaten (National Animal Disease Center, Ames, Iowa).
* Initiated in our laboratory.
* Purchased from the American Type Culture Collection (ATCC), Rockville, Md.
* Obtained from the Naval Biomedical Research Laboratory (NBRL), Oakland, Calif., through the courtesy of Dr. W. A. Nelson-Rees.
* Initiated by cocultivation of TbbLu cells with AJ-147 BC cells.
* Initiated by cocultivation of TbbLu cells with FLS·NI-1228 cells.

Table 1
Cell cultures used

In Vitro Propagation of BLV

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elsewhere (17) using poly(rA)-oligo (dT)_{12-18} as the synthetic template primer and Mg^{2+} as the divalent cation. Twenty to 40 ml of culture fluid were clarified by centrifugation at 12,000 \times g for 10 min and then centrifuged through 20% glycerol in 0.05 M Tris-HCl (pH 7.8), at 100,000 \times g for 90 min at 4°. The resulting virus pellet was resuspended in 0.1 ml of virus-diluting buffer [0.05 M Tris-HCl (pH 7.8):0.1 M NaCl:1 mM dithiothreitol] containing 0.05% Triton X-100. The virus suspension (0.01 ml) was added to a 0.04-ml reaction mixture containing 0.05 M Tris-HCl (pH 7.8), 0.08 M KCl, 2 mM dithiothreitol, 10 mM magnesium acetate, 0.02 A_{260} unit polyriboadenylate and 0.02 A_{260} unit of oligo (dT)_{12-18} (Collaborative Research, Waltham, Mass.). 1 \mu M \text{[H]}TTP (45,000 cpm/pmole) (New England Nuclear, Boston, Mass.), and 0.05% Triton X-100. The results are expressed as cpm of \text{[H]}TTP incorporated into the polydeoxythymidylate product using 60 min incubation at 37°. Activity 3 or more times higher than the blank cpm was considered to be due to viral enzyme.

Serological Tests. Details of the indirect IFA technique using acetone-fixed cells, the immunodiffusion test, the reference sera, and target cells have been described (6-11, 24). Reference BLV Serum SE-276, obtained from a cow with histopathologically confirmed leukemia, was used at the dilution of 1:8 or 1:16. The IFA titer of this serum on BLV-infected cells is 1:256, while at the dilution of 1:8 it is completely negative on BSV-infected cells. Reference BSV Serum SE-354 was collected from a normal cow (BH-128) in leukemia-free and BLV-free Herd BH (9). This serum is negative in the IFA test with BLV-infected cells and has a titer of 1:256 on target BSV-infected cells (6, 23, 24). Fluorescein-conjugated reference sera for IBR, PI_{1}, and BVDV were obtained from the National Animal Disease Laboratory (Ames, Iowa) through the courtesy of Dr. Donald Webert (School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa.). Dr. Van der Maaten supplied us with reference serum (SE-272) for the bovine Visna-like R-29 virus. This serum was collected from a calf inoculated with bovine BC cells infected with R-29 (32). After absorption with uninfected bovine lymphocytes, Serum SE-272 reacts strongly in IFA test with R-29-infected cells and is completely negative on uninfected bovine cells.

Target BLV-infected cells were obtained from NBC-13 cultures in which 15 to 30% of the cells contained viral BLV antigen. Target BSV-infected cells were prepared from the BS-2 cell culture. Acetone-fixed bovine embryonic kidney cells infected with IBR, PI_{1}, or BVDV were kindly supplied by Dr. Donald Webert.

The method for preparing antigen from BLV pellets by ether treatment has been reported previously (8-10).

Electron Microscopy. Cells collected from confluent monolayers by scraping, and high-speed pellets obtained from clarified supernatant fluids were processed as described earlier (14, 24, 28). The specimens were fixed with 3% glutaraldehyde, postfixed with osmium tetroxide, stained \textit{en bloc} with 0.5% uranyl acetate, dehydrated with ethanol, and embedded in Maraglas. Thin sections were stained with uranyl acetate and lead citrate and examined with a Siemens 1A electron microscope (Siemens Corp., Iselin, N. J.).

Chromosomal Analysis. The procedure of Hitotsumachi et al. (20) was followed. Semiconfluent monolayer cell cultures were treated with Colcemid (0.6 \mu g/ml medium; Grand Island Biological Company, Grand Island, N. Y.) for 2 hr; cells were then harvested by trypsinization, resuspended in 1% sodium citrate, and incubated at 37° for 20 min. They were then fixed and washed with methanol:acetic acid (3:1) and stained with Wright and Giemsa stain. Thirty to 50 metaphase spreads from each culture were examined.

RESULTS

The FLS·NI-1228 cell culture supplied by Dr. Van der Maaten was screened for BLV production shortly after arrival at our laboratory. As determined by both the RDDP assay and electron microscopy, the amount of virus released by this culture was only moderate (15,000 to 35,000 cpm in the RDDP assay) and declined further following subsequent subcultivations. Since the value of the FLS·NI-1228 cells as a virus source is also limited by a slow growth rate, attempts were made to transmit BLV from these cells or from AJ-147 BC cells to other monolayer cultures using cocultivation procedures. All of the cocultivated cultures were screened periodically for the presence of cells with the internal BLV antigen applying the IFA technique, for BLV production by the RDDP assay and electron microscopy, and for syncytia formation.

As shown in Table 2, virus production was detected in cultures of rat TblLu, simian DBS, canine FC_{1}Th, and ovine FLS cells soon after they were cocultivated with FLS·NI-1228 cells. The amounts of virus produced in the TblLu culture increased upon further cultivation, reaching high levels. This culture, designated cell line BLV-bat_{1}, is now in its 75th passage and continues to release large amounts of virus. In the FC_{1}Th culture, virus production was moderate and constant during the period of observation. On the contrary, a progressive decline in virus production was noted in the DBS and FLS cells 8 to 12 weeks after cocultivation. Syncytia and cells with the internal BLV antigen were present in all the cultures at every passage tested.

In another series of experiments (results not shown), cultures of TblLu, DBS, FLS, caprine S-743, bovine BESP, and human WI-38 cells were cocultivated with BLV-infected AJ-147 BC cells. The cocultivated TblLu culture, designated as cell line BLV-bat_{2}, was again the only one in which virus production increased progressively and reached high levels. Virus production was low (1,500 to 14,000 cpm in the RDDP assay) but constant in the DBS, S-743, and FLS cultures. BESP and WI-38 cultures produced very low levels (less than 1,500 cpm in the RDDP assay) of virus in the 1st 2 passages after cocultivation and were then completely negative. Syncytia and cells with the internal BLV antigen were also detected consistently in all cultures, regardless of whether or not they released virus. The cytopathic effect appeared earlier and was most abundant in the BESP and WI-38 cells.

Table 3 shows the results of some experiments in which

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* D. C. Graves and J. F. Ferrer, manuscript submitted for publication.
Table 2
Propagation of BLV in monolayer cell cultures infected by cocultivation

<table>
<thead>
<tr>
<th>Host cells (species)</th>
<th>BLV-infected cell inoculum</th>
<th>Assay</th>
<th>Results at following times after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-4 wk</td>
</tr>
<tr>
<td>TblLu (bat)</td>
<td>FLS-N1-1228</td>
<td>RDDP*</td>
<td>20,090</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA*</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia*</td>
<td>+++</td>
</tr>
<tr>
<td>DBS (simian)</td>
<td>FLS-N1-1228</td>
<td>RDDP</td>
<td>20,038</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia</td>
<td>+</td>
</tr>
<tr>
<td>FCLfTh (canine)</td>
<td>FLS-N1-1228</td>
<td>RDDP</td>
<td>29,810</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia</td>
<td>NT</td>
</tr>
<tr>
<td>FLS (ovine)</td>
<td>FLS-N1-1228</td>
<td>RDDP</td>
<td>9,294</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Performed on 20 to 40 ml of supernatant culture fluids. Results are expressed as the cpm of [3H]TMP incorporated into polydeoxythymidylate product during incubation for 60 min at 37°C. Values were corrected by subtracting 3 times the cpm of the blank.

The percentage of positive cells in IFA test with reference BLV serum was determined by examining at least 200 cells.

Results are expressed as the approximate percentage of the monolayer sheet showing syncytia (+, 1 to 10%; ++, 10 to 25%; +++ >30%).

'S NT, not tested.

Table 3
Transmission of BLV to monolayer cell cultures by inoculation with cell-free virus preparations

<table>
<thead>
<tr>
<th>Host cells*</th>
<th>Inoculum</th>
<th>Assay</th>
<th>Results at following times after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-8 wk</td>
</tr>
<tr>
<td>TblLu</td>
<td>CFP from BLV-bat (passage 27)</td>
<td>RDDP*</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia*</td>
<td>+</td>
</tr>
<tr>
<td>TblLu</td>
<td>CFF from BLV-bat (passage 27)</td>
<td>RDDP</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia</td>
<td>+</td>
</tr>
<tr>
<td>DBS</td>
<td>CFF from BLV-bat (passage 41)</td>
<td>RDDP</td>
<td>3,760</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia</td>
<td>+</td>
</tr>
<tr>
<td>PT001Sp</td>
<td>CFF from BLV-bat (passage 36)</td>
<td>RDDP</td>
<td>0</td>
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<td></td>
<td></td>
<td>IFA</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syncytia</td>
<td>+</td>
</tr>
</tbody>
</table>

* The monolayer cell cultures were pretreated with DEAE-dextran for 30 min prior to inoculation.

The percentage of positive cells in IFA test with reference BLV serum was determined by examining at least 200 cells.

Results are expressed as the approximate percentage of the monolayer sheet showing syncytia (+, 1 to 10%; ++, 10 to 25%; +++ >30%).

we attempted to transmit BLV to monolayer cell cultures by means of cell-free preparations. Fourteen to 18 weeks after inoculation with CFP or CFF fluids from the BLV-bat lines, the TblLu cell cultures showed a progressive increase in virus production. Syncytia and fluorescent cells were present at several passages before the cultures began to release detectable amounts of virus. In a more recent experiment fluorescent cells and syncytia were detected in cultures of DBS cells or chimpanzee spleen PT001Sp cells inoculated with BLV-containing CFF. Low levels of RDDP activity were...
also found in the infected DBS culture. The CFF preparations used in this experiment were obtained from cell line BLV-bat, at later passages than those used to infect the TblLu cells. Thus, since virus production increased progressively in this cell line, the finding that the DBS culture showed detectable levels of RDDP earlier than did the TblLu culture was most probably due to a higher concentration of BLV in the inoculum rather than to greater permissiveness of the simian cells.

S-743, FLS, WI-38, and BESP cultures were also inoculated with cell-free BLV preparations obtained from either cell line BLV-bat, or short-term cultures of AF-147 BC cells, and they were maintained for 8 to 12 weeks (results not shown). As in the experiments described above, syncytia were seen earlier than fluorescent cells in all cases. The cytopathic effect was most extensive in the inoculated BESP and WI-38, cells, which did not release virus in detectable quantities. The inoculated S-743 and FLS cells began to produce virus after several passages, but only in moderate and low amounts, respectively.

Cells as well as high-speed virus pellets obtained from the cultures infected by cocultivation with FLS · NI-1228 or AJ-147 BC cells or by direct inoculation with BLV were examined by electron microscopy. Particles with the characteristic morphology of BLV were detected in all the cell samples (Fig. 1A) and high-speed virus pellets (Fig. 1D) obtained from cultures that were positive in the RDDP assay, but they were most abundant in those obtained from the BLV-bat cell lines and FClTh cultures. Virus particles budding at the cell surface were also observed frequently (Fig. 1, B and C). Also in agreement with the results of the RDDP assay, the infected syncytial-positive BESP and WI-38 cells showed only a few virus particles in the 1st 2 passages and thereafter were completely negative. Particles resembling BSV, P13, IBR, BVDV, and R-29 virus were not found in any of the BLV-infected cultures. Repeated IFA tests with appropriate reference sera also failed to show the presence of cells with the antigens of these viruses in any of the cultures used in this study. The results of a representative IFA analysis are summarized in Table 4.

BLV pellets were obtained from the BLV-producing cultures, treated with ether and tested by immunodiffusion against reference BLV serum. As shown in Fig. 2, these pellets give a continuous line of precipitation showing reactions of complete identity with the reference BLV antigen obtained from cell line NBC-13. None of the BLV pellets precipitated with the reference BSV serum (Fig. 3).

All the uninfected cultures used in this study were negative for syncytial cells; for cells reacting in the IFA test with reference sera for BLV, BSV, P13, IBR, BVDV, or R-29 virus; for virus particles; and for RDDP activity. Cell line BLV-bat, was also tested by Dr. Levine for Mycoplasma contamination and was found to be negative.

It was important to ascertain whether transmission of the virus from the FLS · NI-1228 or AJ-147 BC cells to the host cells had occurred in the cocultivated TblLu, DBS, and FClTh cultures. Thus, chromosome analyses were conducted in order to determine whether ovine or bovine cells were present in these cultures when they were actively producing virus. Thirty to 50 metaphase plates obtained from each culture at passages 20 to 30 were examined. Neither ovine nor bovine karyotypes were observed in any of the samples. The chromosomal constitutions of the cocultivated cultures and the corresponding uninfected cultures were similar.

**DISCUSSION**

Extending previous findings (6, 17) we describe the persistent infection of monolayer cell cultures of human, rhesus monkey, chimpanzee, canine, bovine, ovine, caprine, and bat origins with BLV. At every passage tested, all infected cultures, regardless of whether or not they produced complete virus, showed cells with internal BLV antigen and syncytia. Previous results (6) have demonstrated that BLV can induce rapid syncytia formation in the same cultures that were used in the present study. Despite repeated immunological analyses and electron microscopic examinations, no BSV, IBR, P13, BVDV, R-29 virus, or any other viral agents were found in either the FLS · NI-1228, the AJ-147 BC cells, or the monolayer cultures before or after infection with BLV. This indicates that the syncytia observed in the infected cultures were induced by BLV.

Of the monolayer cultures tested the bat TblLu cell line was found to be the most permissive for BLV replication. Upon cocultivation or direct inoculation with cell-free BLV preparations, the bat cells showed a progressive increase in virus production that reached high levels. One of the bat cell cultures infected by cocultivation with FLS · NI-1228 cells (cell line BLV-bat) has been maintained for over 1 year and continues to release large amounts of virus. Since this culture is also free of adventitious agents, is easy to maintain, and contains only small amounts of cell debris, it provides a suitable source of BLV for studies on its antigenic, biochemical, and biological properties.

The inoculated canine cultures release moderate but constant levels of virus. In the simian and caprine cultures virus replication began to decrease 2 to 3 months after cocultivation but at the present time remains constant. A few passages after their arrival at our laboratory, the FLS · NI-1228 cells maintained by themselves produced a constant low level of virus. Adding fresh FLS cells to this culture led only to a moderate and transient enhancement of virus production. The limited ability of infected ovine cells to produce BLV has also been noted by Van der Maaten et al. (34) and...
M. Lieber (personal communication). Ressang et al. (27) reported on the propagation of BLV in FLS cells but did not provide information on the amounts of virus produced.

As described previously (6) and confirmed in the present studies, after infection with BLV by either cocultivation with AJ-147 BC cells or by direct inoculation with virus preparations, WI-38 and BESP cells show very low levels of virus and for only 1 or 2 passages. Subsequently, these cultures did not release detectable amounts of virus although they remained infected, as indicated by the continuous presence of extensive syncytia and cells with the major internal BLV antigen. The same results were obtained with other bovine monolayer cultures inoculated with BLV. Furthermore, we have found that cells with BLV antigen and syncytia are continuously present in long-term cultures derived from the tumor tissues of leukemic cattle; however, such cultures produce virus in low quantities and for only a few passages (unpublished observations). Although the presence of BLV particles in bovine monolayer cultures has been reported by others (27, 34), neither the time during which the virus was produced nor the amounts of virus were mentioned. Studies from this laboratory have also shown that, under conditions of normal maintenance, continuous cultures of BLV-infected lymphoid cells (NBC cell lines) do not produce complete virus (10, 11, 14). Thus, as in the case of the endogenous C-type viruses of other species (1, 29, 31), BLV appears to have very limited ability to replicate in cells of the homologous host. However, results of nucleic acid hybridization studies indicate that BLV is not an endogenous bovine virus (4).

The factors that are responsible for inhibiting the replication of BLV have not been examined. One possible explanation for the decline in virus production with passage in the ovine and caprine cultures is that complete virus is synthesized by a subpopulation of infected cells with a relatively slower growth rate than the rest of the cell population. This possibility is supported by preliminary results of cloning experiments with cell line BLV-bat. It is also conceivable that a virus inhibitor is produced in these cultures. Cornefert-Jensen et al. (5) have detected low levels of interferon in monolayer cultures of WI-38 or bovine cells cocultivated with lymphoid cells from leukemic cattle.

The results of chromosome analyses support the conclusion that in the cocultivated bat, canine, simian, and caprine cultures the virus was transmitted to and is produced mainly or exclusively by the host cells. Ovine karyotypes were not found in any of the 30 or more metaphase plates prepared from each of the cultures cocultivated with FLS·NI-128 cells. Since, as determined by the IFA test, at least 20 to 50% of the cells in these cultures were infected with BLV, it is unlikely that the virus was produced by remaining FLS·NI-1228 cells. The progressive disappearance of the FLS·NI-1228 cells upon passage of the mixed cultures was expected, because the growth rate of these cells is considerably slower than the growth rate of the host cells. Cytological examination of the cultures cocultivated with bovine BC cells have indicated that these cells do not become established in the monolayer and are removed with the 1st medium changes (6). Consistent with this observation is the fact that cells with bovine karyotype were not found in the virus-producing cultures inoculated with AJ-147 BC cells.

The finding that BLV can be transmitted to other cells by either cocultivation or direct inoculation provides definitive evidence of the infectious nature of this virus.

Both the free and budding BLV particles found in the monolayer cell cultures were indistinguishable from those previously observed in short-term or long-term cultures of bovine lymphoid cells (11, 14, 28). Calafat et al. (3) reported the presence of budding particles with electron-dense nucleoids in BLV-producing bovine lymphocytes. Such particles were never seen in the monolayer cell cultures described here or in the bovine lymphoid cultures studied in our laboratory. Calafat et al. (3) observed these atypical budding particles only in a few instances and in short-term lymphoid cell cultures in which the presence of viruses other than BLV had not been ruled out. Thus, the conclusion that these particles correspond to BLV is not warranted.

As determined in immunodiffusion tests, the BLV particles produced by the monolayer cultures possess the internal virion antigen previously identified in BLV particles obtained from the NBC cell lines and from short-term cultures of bovine BC cells (8–13, 23, 24). In addition, virus neutralization and absorption studies have demonstrated that the virus particles released by the BLV-bat cell lines and the AJ-147 BC cells share envelope antigens and the ability to induce syncytia formation in monolayer cultures of various origins (6). Thus, it appears that neither the antigenic nor the biological properties of BLV have changed as a result of its propagation in heterologous cells.

The fact that BLV can infect human, chimpanzee, and rhesus monkey cell lines deserves particular attention in terms of its potential as a human biohazard.

ACKNOWLEDGMENTS

We thank Dr. R. R. Marshak for his support and encouragement, Dr. C. Diglio for his help in cytological examinations of the cultures, Dr. M. Lieber for helpful suggestions, and Dr. E. M. Levine for testing the cultures for Mycoplasma. The skillful technical assistance of A. Brundige and V. Baliga, and the excellent secretarial help of B. Blevins are also gratefully acknowledged.

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Fig. 1. Electron micrographs of the BLV released by cell line BLV-bat, a, an aggregate of mature extracellular virus particles. × 81,000. b and c, virus particles budding from the cell membrane. × 84,000. d, a cell-free pellet obtained by ultracentrifugation (100,000 × g, 90 min) of preclarified (10,000 × g, 15 min) supernatant culture fluid. × 44,000.

Fig. 2. Immunodiffusion analysis of virus pellets from various BLV-infected monolayer cell cultures with reference BLV Serum Se-276 (center well). Peripheral wells contain ether-treated virus pellets from cell line BLV-bat (Well 1), cell line BLV-bat2 (Well 2), a BLV-infected DBS cell culture (Well 3), and a BLV-infected FCTh cell culture (Well 4). Well 5 contains reference internal BLV antigen (obtained from cell line NBS-13).

Fig. 3. Immunodiffusion analysis of virus pellets from various BLV-infected monolayer cell cultures with reference BSV Serum Se-354 (center well). Peripheral wells contain ether-treated virus pellets from cell line BLV-bat (Well 1), cell line BLV-bat2 (Well 2), a BLV-infected DBS cell culture (Well 3), and a BLV-infected FCTh cell culture (Well 4). Well 5 contains reference BSV antigen (obtained from BSV-infected cell cultures BS-2).
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