[CANCER RESEARCH 36, 1056-1067, March 1976]

Induction of Syncytia by the Bovine C-type Leukemia Virus[1]

Clement A. Diglio[2] and Jorge F. Ferrer

Section of Viral Oncology, Comparative Leukemia Studies Unit, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348

SUMMARY

Bovine buffy coat cells infected with the bovine leukemia virus (BLV) induce syncytia formation in human diploid embryonic lung cells as well as in monolayer cell cultures of bovine, simian, ovine, bat, and caprine origin, but not in mouse fibroblast cells, normal rat kidney cells, or RSV-transformed rat cells. Syncytia were not observed in diploid embryonic lung cells inoculated with bovine buffy coat cells free of BLV. The syncytia-induction effect is associated with the synthesis of complete BLV by the buffy coat cells and is independent of whether these cells are viable, disrupted, normal, or malignant.

Cell-free preparations of BLV and density gradient-purified virus also induce syncytia when added directly to diploid embryonic lung cells and to bovine, bat, and caprine monolayer cell cultures.

Ether treatment, ultraviolet light irradiation, heating, freezing, and thawing destroy the syncytia-inducing activity of BLV. This activity is also neutralized when the virus is incubated with sera containing antibodies to BLV, but not when incubated with sera free of these antibodies or reference serum for the foamy-like bovine syncytial virus. Several other lines of evidence rule out the possibility that this virus or other bovine viruses are responsible for the syncytia-inducing phenomenon described here.

BLV antigen was consistently detected by the immunofluorescent test in the syncytia-positive monolayer indicator cultures. However, syncytia formation was not necessarily associated with BLV production by the indicator cells.

The ability to induce syncytia in monolayer cultures of nontransformed cells distinguishes BLV from all the known C-type leukemia viruses.

INTRODUCTION

Cornefert-Jensen *et al.* (4) showed that when monolayers of human (WI-38 cells),[3] or of bovine cells (EBTr, BEK) were cocultivated with long-term cultures of bovine leukemic cells (NBC cell lines) or freshly obtained peripheral blood lymphocytes from leukemic cattle, syncytia formation, cytoplasmic vacuolation, and nuclear abnormalities were consistently observed in the monolayer cells. Except in 1 case, this alteration was not seen in the monolayers cocultivated with lymphocytes from normal cattle. The effect was assumed to be due to a virus that was tentatively designated as the Jensen BSV. The only evidence for the existence of this agent was the occasional observation of C-type particles in a few of the syncytia-positive cultures. However, all efforts to isolate the virus and transmit the CPE by cell-free supernatant fluids, cell-free homogenates, or by parabiotic culture techniques were unsuccessful. Cornefert-Jensen *et al.* (5) also demonstrated that the formation of syncytia could be inhibited if, prior to cultivation with the monolayer cells, the bovine lymphocytes were incubated with sera from leukemic cows. A seroepidemiological survey showed that syncytia-inhibiting antibodies were prevalent among multiple-case herds and were rare among leukemia-free herds.

Following these findings, an indigenous bovine virus, morphologically similar to the C-type leukemia viruses, was identified in short-term cultures of BC cells from cattle with leukemia or PL, as well as in the NBC cells lines (10, 24, 33). Seroepidemiological studies (8, 9, 11, 25, 27) and transmission experiments (27) indicate that this virus is the causative agent of bovine leukemia. Therefore, the virus has been designated as the putative BLV.

Since BLV is detected in the same cells (NBC) cell lines and peripheral lymphocytes of leukemic cattle) in which Cornefert-Jensen *et al.* (4) had found syncytia-inducing activity, it seems possible that this virus is responsible for the induction of the syncytia. This was suggested further by the fact that, as in the case of the syncytia-inhibiting antibodies (5), fluorescent antibodies to BLV (8, 11) were found to be prevalent in multiple-case herds and rare or absent in leukemia-free herds.

The present report demonstrates that BLV-infected bovine lymphoid cells, as well as BLV itself, induce syncytia in several monolayer cell cultures. The data indicate that infection of the indicator cells is required for syncytia induction.

Received July 9, 1975; accepted December 4, 1975.

[1] This work was supported in part by USPHS Grant 1-PO1-CA-14193-02, Pennsylvania Department of Agriculture Grant ME-4, and USDA Cooperative Agreement 12-14-100-10,675(45).

[2] Postdoctoral Fellow on USPHS Grant 5-TO1-CA-05097-13.

[3] The abbreviations used are: WI-38 cells, human diploid embryonic lung cells; EBTr, embryonic bovine tracheal cells; BEK, bovine embryonic kidney cells; NBC, New Bolton Center; BSV, bovine syncytia virus; CPE, cytopathic effect; BC, buffy coat; PL, persistent lymphocytosis; BLV, bovine leukemia virus; A-204, human rhabdomyosarcoma cells; DBS-FRhL-1, fetal rhesus lung cells; FCf_2th, fetal canine thymus cells; Tb1Lu, bat lung cells; S-743, mountain goat cells; FLS, fetal lamb spleen cells; F-771, human skin cells; NRK, normal rat kidney cells; 3T3, mouse fibroblast cells; RSV, Rous sarcoma virus; XC, RSV-transformed rat cells; BESP, bovine embryonic spleen cells; BS-1, cell culture derived from a bovine liposarcoma; Bth, bovine thymus cells; CEHC-I, calf embryo heart cells; S-7, sheep thymus cells; R900F, normal human skin cells; MEM, Eagle's minimal essential medium; CSI, calf serum inactivated (56° for 30 min); FSI, fetal calf serum inactivated (56° for 30 min); IFA, immunofluorescent antibody; PI_3, parainfluenza-3 virus; IBR, infectious bovine rhinotracheitis virus; BVD, bovine viral diarrhea virus; RDDP, RNA-dependent DNA polymerase; PBS, phosphate-buffered saline (0.15 M NaCl:0.0061 M $Na_2HPO_4 \cdot 7H_2O$:0.0038 M KH_2PO_4), pH 7.2; PHA, phytohemagglutinin.

MATERIALS AND METHODS

Animals

The following animals were used in this study: 10 cows with histologically confirmed leukemia, 7 referral cases and 3 from multiple-case study herds BF and BG; 6 cows with PL and 2 nonlymphocytotic animals from multiple-case herd BF; and 12 nonlymphocytotic cattle from leukemia-free study herds BI and BH. The characteristics of the study herds and the criteria for defining PL have been reported elsewhere in detail (1, 2, 8, 20, 21).

Monolayer Cell Cultures

Monolayer cultures used as indicators in mixed cell culture experiments are listed in Table 2. Human A-204, simian DBS-FRhL-1, canine FCf_2th, bat Tb1Lu, and caprine S-743 cell cultures were obtained from Dr. M. Lieber (Meloy Laboratories, Springfield, Va.). Lamb FLS cells were supplied by Dr. J. M. Van der Maaten (National Animal Disease Laboratory, Ames, Iowa). Human WI-38 and human F-771 cells free of *Mycoplasma* were obtained from the American Type Culture Collection (Rockville, Md.). Rat NRK cells were obtained from Dr. K. Chang (NIH, Bethesda, Md.). Mouse 3T3 fibroblast cells and RSV-infected nonproducing XC rat cells were provided by Dr. R. Bassin (National Cancer Institute, NIH, Bethesda, Md.). The remaining monolayer cultures that are listed in Table 2 were initiated in our laboratory. The spleen from a 3-month-old bovine embryo was used to establish the BESP monolayer culture that has been maintained for 20 passages and consists of both epithelial- and fibroblast-like cells. This culture has been tested for *Mycoplasma* by Dr. E. M. Levine (The Wistar Institute, Philadelphia, Pa.) and was found negative. The monolayer cell culture designated BS-1 was derived from a bovine liposarcoma and consists of normal-appearing, regularly arranged, contact-inhibited fibroblastic cells. Fibroblastic cell cultures Bth-287, Bth-434, and Bth-469 were initiated with thymus cells of calves in a leukemia-free herd. The heart of a calf embryo in a leukemia-free herd was used to establish the fibroblastic monolayer culture CEHC-I. Fibroblastic cell culture S-7 was derived from the thymus of a newborn sheep. Cell line R900F was established from normal human skin and kindly provided by our associate, Dr. Kuang-dong Wuu.

The same general procedure was used to initiate these cultures. In brief, tissue fragments were minced with sharp scissors and trypsinized. The cells were washed and seeded in 75-sq cm Falcon tissue culture flasks with 25 ml of MEM or Roswell Park Memorial Institute Medium 1640 supplemented with 10 to 20% CSI or FSI, penicillin (100 units/ml), and streptomycin (100 μg/ml). Upon confluency, the cells were dispersed with a mixture of 0.05% trypsin and 0.06% EDTA and subcultured. All these monolayer cultures as well as cell cultures obtained from other laboratories were maintained in MEM supplemented with 10 to 20% FSI, with the exception of CEHC-I, which was maintained in MEM supplemented with 10% CSI.

Lymphoid Cells and Lymphoid Cell Cultures

BC cells were isolated by lysing the erythrocytes by hypotonic shock and cultured *in vitro* following procedures previously reported in detail (33).

The BLV-infected BC cells were obtained from a cow (AJ-147) with PL in multiple-case study herd BF. These cells were used either immediately after they were isolated or after storage in liquid nitrogen with 20% dimethyl sulfoxide. Following *in vitro* culture for 48 to 72 hr, 30 to 50% of the AJ-147 BC cells showed BLV particles as determined by electron microscopy as well as the BLV antigen as detected by the IFA technique on acetone-fixed cells with reference BLV serum (9, 33). The foamy-like BSV described by Malmquist *et al.* (19) was not found in uncultured or cultured AJ-147 BC cells despite extensive and repeated electron microscopic examination and IFA testing with reference BSV serum. In addition, no antibodies to BSV were found in the serum of AJ-147, further indicating that this animal is BSV free. The AJ-147 BC cells were also negative for the maedi-like bovine virus (R-29) described by Van der Maaten *et al.* (35), and for PI_3, IBR, and BVD viruses by both electron microscopy and IFA tests with appropriate reference sera (Table 1).

Cell line NBC-13 was established from peripheral lymphocytes of a cow with leukemia and is routinely maintained as a suspension culture in McCoy's 5A modified medium supplemented with 10 or 20% FSI. Details on the derivation, maintenance, and characteristics of BLV production in this cell line have been reported previously (9, 10, 15, 17). In periodic tests this cell line was found to be consistently free of BSV, R-29, PI_3, IBR, and BVD viruses.

Virus Sources and Virus Preparations

Cell-free preparations of BLV were obtained from the supernatant fluids of short-term cultures of AJ-147 BC cells and cell lines BLV-bat_1 and BLV-bat_2 that were established by infecting the bat monolayer cell culture Tb1Lu with BLV. As determined by both electron microscopy and RDDP assay, these cultures release the virus abundantly and consistently. Immunological analyses using IFA, IFA absorption, immunodiffusion, and virus neutralization tests have demonstrated that the BLV particles released by the BLV-bat cultures are antigenically indistinguishable from BLV produced by the bovine BC cells and NBC cell lines. Repeated electron microscopic examinations and IFA tests with reference sera have failed to detect BSV, R-29, PI_3, IBR, and BVD viruses in the BLV-producing bat monolayer cultures (16, 22).[4]

Cell-free pellets were obtained by ultracentrifugation (100,000 × *g*, 90 min) of preclarified (10,000 × *g*, 15 min) supernatant fluids of the BLV-releasing cultures. Virus pellets obtained from approximately 500 ml of culture fluid were resuspended in 3 ml of MEM and 10% FSI and filtered through a 0.45-μm Nalgene filter. Cell-free filtrates were prepared by filtering supernatant fluid from cell line BLV-

[4] D. C. Graves, and J. F. Ferrer. Bovine Leukemia Virus. I. *In Vitro* Transmission and Propagation in Monolayer Cell Cultures, submitted for publication.

Table 1
Reference bovine sera
Immunofluorescence tests on acetone-fixed cells.

Serum	Serum donor	Target cells infected with					
		BLV[a]	BSV[b]	R-29[c]	PI_3[d]	IBR[d]	BVD[d]
27-125[e]	Regression case of bovine leukemia	+ (256)[f]	+ (256)	–	–	–	–
Se-276	Leukemic cow	+ (256)	±	–	–	–	–
Se-362	Lymphocytotic cow (BF-117) in multiple-case Herd BF	+ (16)	–	–	–	–	–
Se-354	Normal cow (BH-128) in leukemia- and BLV-free Herd BH	–	+ (256)	–	–	–	–
Se-271[g]	Cow (B3554J) with fibrosarcoma	–	+ (256)	–	–	–	–
Se-344[h]	BSV-infected cow	–	+ (128)	–	+ (2)	–	–
Se-272[h]	Calf experimentally infected with R-29	–	–	+	–	–	–

[a] NBC-13 cells (see Refs. 6 to 12, 14).
[b] BESP cells infected by cocultivation with BC cell of cow BH-116 in leukemia- and BLV-free Herd BH.
[c] R-29-infected BESP cells were supplied by Dr. M. J. Van der Maaten (National Animal Disease Laboratory, Ames, Iowa).
[d] Supplied by Dr. D. Webert (School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa.).
[e] See Refs. 6 to 11, 21.
[f] Numbers in parentheses, reciprocal of the titer.
[g] See Ref. 5.
[h] Supplied by Dr. M. J. Van der Maaten. Se-272 was obtained from a calf that received an injection of R-29-infected bovine cells. This serum was absorbed with a homogenate of BLV-free bovine BC cells to remove isoantibodies (see Ref. 34).

bat_2 through a 0.45-μm Nalgene filter. A density gradient-purified BLV preparation was obtained from 750 ml of supernatant culture fluid from cell line BLV-bat_2. The culture fluid was clarified (10,000 × g, 10 min) and the virus was pelletized by centrifugation at 100,000 × g for 90 min. The virus pellet was resuspended in 0.01 M Tris:0.1 M NaCl:0.001 M EDTA buffer, pH 7.2, and centrifuged in a discontinuous sucrose gradient (20 to 50%) for 2.5 hr. The band collected at the 50% interface of this gradient was centrifuged in a continuous sucrose gradient (15 to 55%). A well-defined band was observed in the region of the gradient corresponding to a density of 1.15 g/ml. Electron microscopic examination of this band revealed numerous characteristic C-type particles. Vesicle or structures suggesting the presence of other viruses were not seen in this band.

The source of BSV was a stock of BC cells obtained from a Holstein cow with a fibrosarcoma (Case B3554J). Characteristic BSV particles and BSV antigen were readily detectable in short-term cultures of B3554J BC cells as well as in BESP cultures cocultivated with these cells. The serum of Cow B3554J reacted strongly (titer of 1:256) in the IFA test with target acetone-fixed BSV-infected cells but was negative with target BLV-infected cells (Ref. 6; Table 1). BSV-infected cells were also obtained from a cow (BH-116) in leukemia- and BLV-free Study Herd BH. This animal also had fluorescent antibodies to BSV (titer of 1:64), and large numbers of BSV particles were seen in cultures from its BC cells.

PI_3 (hemagglutination titer of 1:16 on guinea pig RBC), BVD (titer of 10^2 plaque-forming units per ml on BEK cells), and IBR (titer of 10^{10} plaque-forming units per ml on BEK cells) viruses were obtained through the courtesy of Dr. Donald Webert (Section of Epidemiology and Public Health, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa.).

Syncytia Assay

Inoculation with BC Cells. Monolayer indicator cells were seeded into either 75-sq cm Falcon tissue culture flasks or 60-mm Falcon tissue culture dishes at a cell concentration that resulted in a confluent sheet after 5 to 8 days. Twenty-four hr later, viable BC cells were added to the monolayer culture (50×10^6/75-sq cm Falcon flask or 5×10^6/60-mm Falcon dish). The cell mixture was incubated at 37° and after 48 hr the unattached cells were removed by aspiration. The monolayer was washed with Hanks' balanced salt solution and then complete fresh medium was added. Upon con-

fluency the cells were washed with PBS, pH 7.2, fixed with methanol, stained with Giemsa, and examined for cytological alterations and/or dispersed with trypsin and EDTA and subcultured. At each passage, the cells were also seeded in either 25-sq cm Falcon flasks or in Leighton tubes containing coverslips that, upon confluency, were fixed, stained, and examined for CPE.

Inoculation with Cell-free Virus Preparations. Monolayer indicator cells were seeded as described above, and 24 hr later the monolayer was treated for 30 min with complete medium containing DEAE-dextran, 25 μg/ml. The monolayer was then washed with Hanks' balanced salt solution, inoculated with the BLV preparations, and incubated for 2 hr at 37°. Subsequently, complete medium was added and the cultures were reincubated. Upon confluency, the monolayers were subcultured and handled in the same manner as those inoculated with BC cells.

BC Cell Homogenates

Approximately 100 × 10^6 AJ-147 BC cells either not cultured or cultured for 48 hr in MEM 20% FSI, were washed with MEM, resuspended in 6 ml of cold distilled water, and disrupted by Dounce homogenization for 15 min on ice. As determined by the trypan blue exclusion method, no viable cells remained in these homogenates. The homogenates were then centrifuged at 10,000 × g for 10 min, and the supernatant fluids and pellets were collected separately. The pellets (precipitable fraction) were resuspended in 10 ml of MEM 10% FSI and the supernatant fluid was diluted to 10 ml with MEM 10% FSI. Duplicate 5-ml samples of each of these preparations were then tested for syncytia-inducing activity on WI-38 cells in 75-sq cm Falcon flasks.

Reference Sera

The origin and characteristics of the reference sera used are given in Table 1. The γ-globulin fractions from sera Se-276 and 27-125 were obtained in a partially purified form by 3 consecutive precipitations with ammonium sulfate. Residual sulfate was removed by dialysis and the γ-globulin was resuspended in distilled water to a final concentration of 11 mg of protein per ml.

Inhibition of the Syncytia-forming Activity of BLV

Serum Neutralization. Filtered cell-free BLV from culture fluids of cell line BLV-bat_2 was incubated at 37° for 1 hr with an equal volume of heat-inactivated (56° for 30 min) undiluted serum or with a γ-globulin solution containing 11 mg of protein per ml. The mixture was then inoculated onto duplicate 60-mm Falcon dishes (2 ml/dish) containing BESP indicator cells. In the experiments in which AJ-147 BC cells were used as the syncytia-inducing inoculum, a suspension containing 1 × 10^6 viable BC cells per ml was incubated for 1 hr at 37° with an equal volume of heat-inactivated undiluted serum. Two ml of the mixture were then inoculated onto 60-mm Falcon dishes containing WI-38 indicator cells. Each BC cell-serum mixture was tested in duplicate.

UV Irradiation. Five ml of culture fluid of BLV-bat_2 were added to a 60-mm Falcon dish that was placed 56 cm from a Letheray germicidal lamp (120 V-60 cycle, UV output 2537A, 15 watts) for 15 to 20 min. The irradiated BLV preparation and an unirradiated aliquot of the same preparation were filtered through a 0.45-μm Nalgene filter and tested on BESP cells.

Freezing and Thawing. Five ml of filtered, cell-free BLV were frozen at −70°, thawed, and then tested on BESP cells, with an unfrozen aliquot of the same preparation as a control.

Heat Inactivation. Five ml of filtered cell-free BLV were heated at 56° for 15 min and then tested on BESP cells. An unheated aliquot of the same preparation served as the control.

Ether Treatment. A cell-free BLV pellet obtained by ultracentrifugation of preclarified cultured medium of cell line BLV-bat_2 was resuspended in 2 ml of MEM, mixed with 4 ml of ether, and stirred for 1 hr at room temperature. The remaining ether was then removed by vacuum evaporation. The mixture was brought up to a volume of 5 ml by adding MEM and was centrifuged at 100,000 × g for 90 min. The resulting pellet and supernatant fluid were collected separately. The pellet was resuspended in 2 ml of MEM. The resuspended pellet and the supernatant fluid were filtered through a 0.45-μm filter and tested for syncytia-inducing activity on indicator BESP cells. A cell-free pellet of BLV obtained from an aliquot of the BLV-bat_2 culture fluid was resuspended in 5 ml of MEM, filtered, and used as a control.

IFA and IFA Absorption Tests

The indirect IFA method on acetone-fixed cells was carried out as described previously (7–9). Target lymphoid cells were fixed a few min after they were added to the wells on Fluoroglide-coated slides. Target cells were obtained from confluent monolayer cultures by trypsin-EDTA treatment, washed once with PBS, adjusted to a concentration of 3 × 10^5 cells/ml of MEM 10% FSI, placed in the wells, incubated for 3 to 4 hr at 37° in a humidified 5% CO_2 incubator, and then fixed.

Target BLV-infected cells were obtained from NBC-13 cultures in which 15 to 30% of the cells showed viral BLV antigen. Target BSV-infected cells were prepared from the 2nd or 3rd passage of syncytia-positive BESP cultures inoculated with BC cells of Cow BH-116 or B3554J.

BESP cells infected with R-29 (35) were obtained through the courtesy of Dr. M. J. Van der Maaten (National Animal Disease Laboratory).

Acetone-fixed BEK cells infected with IBR, PI_3, or BVD viruses were kindly supplied by Dr. Donald Webert.

The IFA absorption test was carried out by mixing reference BLV Serum 27-125 diluted 1:100 (approximately two 2-fold dilutions below its end point) with an equal volume of the antigen preparation or MEM. After incubation at 37° for 1 hr and overnight at 4°, the mixtures were centrifuged at 5000 × g for 15 min. The serum was removed and tested for residual activity by the indirect IFA technique on BLV-infected NBC-13 cells.

The method for preparing antigen from BLV pellets or

extracts of infected cells by ether treatment has been reported previously (8).

Electron Microscopy

Cells for electron microscopic examination were obtained from confluent monolayers by scraping and were processed as described previously (33). In order to ensure that each cell section viewed represented a different cell section, only a single section of each block was examined.

RDDP Assay

The RDDP or reverse transcriptase of BLV was detected by applying the method of Ross *et al.* (31) as modified by Todaro *et al.* (34). The synthetic template primer poly(rA)·oligo(dT)$_{12-18}$ was used and magnesium was substituted for manganese in the reaction mixture since the RDDP of BLV, unlike that of most known mammalian C-type viruses, requires this cation rather than manganese for optimal reactivity (16). Twenty to 40 ml of culture fluid were clarified by centrifugation at 12,000 × *g* for 10 min and then centrifuged at 100,000 × *g* for 90 min at 4°. The resulting virus pellet was resuspended in 0.1 ml of 0.05 M Tris-HCl buffer, pH 7.8, containing 0.10 M NaCl, 1 mM dithiothreitol, and 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; 0.06 M KCl, 2 mM dithiothreitol; 6 mM magnesium acetate, 0.02 A_{260} unit poly(rA) and 0.02 A_{260} unit oligo(dT)$_{12-18}$ (Collaborative Research, Waltham, Mass.); and 1 mM [^{3}H]TTP (60,000 cpm/pmole) (New England Nuclear, Boston, Mass.). The results are expressed as cpm of [^{3}H]TTP incorporated into the poly(dT) product during 60 min incubation at 37°. Activity 3 or more times higher than the blank cpm was considered to be due to viral enzyme.

RESULTS

Syncytia Formation in Monolayer Cell Cultures of Various Origins Cocultivated with BLV-infected Bovine BC Cells. As indicated in Table 2, monolayer cell cultures of human, bovine, ovine, caprine, canine, bat, and simian origins underwent syncytia formation when cocultivated with BLV-infected BC cells from Cow AJ-147. Monolayer cell cultures of murine and rat origin did not.

In the mixed cultures, most inoculated BC cells were removed with the 1st medium change 48 hr after cocultivation; the remaining BC cells became detached from the monolayer soon afterward. Occasionally, a few lymphoid cells were retained within the monolayer but, unlike the results obtained by Cornefert-Jensen *et al.* (4), they did not appear to fuse with other cells to form syncytia.

The CPE noted in the susceptible monolayers was similar to that described by Cornefert-Jensen *et al.* (4). Syncytial cells (cells with 5 or more nuclei) usually began to appear within the 1st 5 days after cocultivation. Cells with 2 to 4 nuclei were occasionally observed in the control uninoculated monolayers, particularly in cell lines A-204 and XC. Therefore, cells with 4 or less nuclei were not counted as syncytial cells. Syncytial and mononucleated cells with cytoplasmic vacuolation and nuclear fragmentation were often seen. Table 3 illustrates the levels of syncytia found in 6 of the monolayer cultures during the 1st 2 passages. BESP and WI-38 cells showed the most extensive CPE and degenerated completely after a few passages unless uninfected BESP and WI-38, respectively, were added. In the other monolayers tested, the numbers and size of syncytia increased during the 1st subcultivations, but fluctuated in subsequent passages. No mitotic figures were ever observed in the syncytial cells.

Fig. 1 shows characteristic syncytial cells in the BESP (Fig. 1*A*) and WI-38 (Fig. 1*B*) cultures, which were used most

Table 2

Susceptibility of monolayer cell cultures of various origins to the syncytia-inducing activity of BLV-infected bovine BC lymphocytes[a]

Monolayer cell cultures		Syncytia formation[b]
Designation	Origin	
BESP	Bovine embryo spleen	+
BS-1	Bovine lyposarcoma	+
Bth-287	Bovine thymus	+
Bth-434	Bovine thymus	+
Bth-469	Bovine thymus	+
CEHC-I	Bovine heart	+
S-7	Ovine thymus	+
FLS	Ovine spleen	+
S-743	Caprine ovary	+
FCf$_2$th	Canine thymus	+
WI-38	Human lung	+
A-204	Human rhabdomyosarcoma	+
F-771	Human skin	+
R900F	Human skin	+
Tb1Lu	Bat lung	+
DBS-FRhL-1	Rhesus lung	+
3T3	Murine embryo	−
NRK	Rat kidney	−
XC	Rat sarcoma	−

[a] AJ-147 BC cells (50 × 10^6) were added to a 75-sq cm Falcon flask containing a semiconfluent sheet of the monolayer cell culture.

[b] Cultures were monitored for syncytia (cells with 5 or more nuclei) for 3 passages; +, positive (the number of syncytia observed varied considerably among the different monolayer cell cultures).

Table 3

Effect of subcultivation on the syncytia response of various indicator cells inoculated with BLV-producing BC cells

Approximately 50 × 10^6 viable AJ-147 BC cells, obtained from the same frozen stock, were inoculated into a 75-sq cm Falcon flask containing the indicator cells.

Indicator cells	Degree of syncytia[a]		
	Before subcultivation	Passage 1	Passage 2
BESP	+++	+++	+++
WI-38	++	+++	+++
Tb1Lu	+	++	+++
S-743	+	+	NT[b]
DBS-FRhL-1	+	+	+++
FCf$_2$th	+	+	+++

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

[b] NT, not tested.

extensively in these studies as indicators.

Susceptibility of WI-38 and BESP Cells to the CPE of Common Bovine Viruses Other Than BLV. Because of their susceptibility and rapid response to the syncytia-inducing effect of BLV-infected BC cells, WI-38 and BESP cells were selected as indicators in this study. Therefore, it was of interest to investigate whether these cells were susceptible to the CPE of other viruses widespread in the cattle population.

BC cells infected with BSV and free of BLV were obtained from Cow B3554J and tested following the procedures described above. Each Falcon flask (75-sq cm) containing the indicator monolayer was inoculated with 50×10^6 viable BC cells. No CPE was observed in any of the experiments in which WI-38 cells were inoculated (in 1 case, repeatedly) with the BSV-infected BC cells. In these experiments the indicator cells were monitored for a period of at least 4 weeks. In contrast, the BSV cell inoculum always induced extensive syncytia formation in BESP cells. The syncytial cells began to appear after the BESP cells were subcultured and increased rapidly in subsequent passages. The same cytopathic changes were observed in BESP cells cocultivated with BC cells of 6 cattle that belonged to leukemia- and BLV-free Study Herd BH and had antibodies to BSV. As shown below, the BC cells from these animals did not induce syncytia in WI-38 cells.

BESP monolayers inoculated with BSV-infected BC cells from Cow B3554J or BH-116 were tested by the IFA technique at Passages 2 and 4 with reference BSV serum. Cytoplasmic and nuclear staining, similar to that described by Malmquist *et al.* (19), were seen in a large percentage of the single and syncytial cells in these cultures.

Preparations of PI_3, IBR, and BVD viruses were tested undiluted and in serial 10-fold dilutions. Each 60-mm Falcon dish was inoculated with 0.5 ml of the virus preparation and the cultures were monitored daily for 7 days. Both WI-38 and BESP cultures were highly susceptible to the syncytia-inducing activity of PI_3 virus. After infection with IBR virus, both monolayers underwent extensive cytocidal CPE and degenerated completely within a few days without forming syncytia. A similar CPE was observed in BESP cells infected with BVD virus. On the other hand, this virus did not induce CPE in WI-38 cells.

Correlation between Syncytia-inducing Activity and BLV Production in Bovine BC Cells. In the 1st experiment BC cells were collected from 18 cows that were infected with BLV as determined by the IFA test (8, 12). This group includes 10 leukemic cows and 8 clinically normal animals from multiple-case Study Herd BF (1, 2). Six of the 8 BF cattle had PL. The presence of BLV particles was determined by electron microscopic examination of PHA-treated short-term cultures of BC cells from 17 of these animals. This confirms previous data demonstrating that the presence of IFA antibody to BLV correlates closely with the presence of the virus (8, 12). The effect of PHA in promoting BLV expression in bovine BC cultures has been reported (24, 33).

Control BC cells free of BLV were obtained from 2 leukemic calves and 12 animals belonging to leukemia-free Study Herds BI and BH (2, 7). BH-116, BH-128, and other cattle included in this control group had fluorescent antibodies against BSV, and their BC cells induced syncytial cells and cells with BSV antigen in BESP cultures. All the control animals were negative in the IFA test for BLV infection. In addition, PHA-treated short-term cultures of BC cells from 6 animals (including the 2 leukemic calves) in the control group were tested for BLV particles and found to be negative.

The results (Table 4) show that the BC cells of the 18 cattle infected with BLV induced syncytia in WI-38 cells. Conversely, the CPE was not observed when the indicator cells were cocultivated with BC cells of the control BLV-free cattle.

As reported previously (8, 33), neither BLV nor its antigen can be detected in uncultivated infected bovine BC cells, but both complete virus particles and virus antigen are expressed when these cells are maintained *in vitro* even for a few hr. Thus, the syncytia-inducing activity of the BLV-infected BC cells may be due to the virus released by these cells during the period of cocultivation with the indicator monolayer.

Two experiments were carried out to test this possibility and to examine further the relationship between syncytia-inducing activity and BLV production in the bovine BC cells. The 1st experiment was designed to: *(a)* compare the syncytia-inducing activity of uncultured (nonproducing) *versus* cultured (producing) AJ-147 BC viable cells; and *(b)* determine the effect of the incubation time of the AJ-147 BC cell inoculum with the indicator cells on the syncytia response.

The results summarized in Table 5 show that, after comparable incubation times, cultured BC cells induced significantly higher numbers of syncytia in WI-38 cells than did a double inoculum of uncultured BC cells. The results also show that, in the WI-38 cultures inoculated with uncultivated BC cells, the numbers of syncytia increased as the incubation times were prolonged.

We also compared the syncytia-inducing activities of cell-free homogenates of uncultured *versus* cultured AJ-147 BC cells. The homogenates were prepared from comparable numbers of cells, and both the precipitable and soluble fractions of each of the homogenates were assayed separately and in duplicate on WI-38 cells with an inoculum of 5

Table 4

Correlation between the presence of BLV and syncytia-inducing activity in BC cells

BC cells (50×10^6) were added to a 75-sq cm Falcon flask containing a semiconfluent sheet of indicator WI-38 cells. The cultures were monitored at each passage for syncytia formation.

Source of BC cells	No. of animals tested	Syncytia-inducing activity	
		No.	%
BLV-infected cattle[a]	18	18	100
Uninfected cattle[b]	14	0	0

[a] All animals were positive in the IFA test for BLV. PHA-treated short-term cultures of BC cells of 17 of these animals were also tested for BLV particles and found positive.

[b] All animals were negative in the IFA test for BLV. BLV particles were not found in the PHA-treated short-term BC cultures of the 6 animals examined in this group.

ml/75-sq cm Falcon flask. The results showed that, whereas both fractions of the homogenate of the BC-cultured cells induced large numbers of syncytia, neither fraction of the uncultured cell homogenate was active.

Previous studies have shown that cell line NBC-13 is infected with BLV but that the expression of complete virus and virus antigen varies considerably depending upon the tissue culture conditions (9, 10, 14). Thus, in order to further examine the correlation between BLV production and syncytia-inducing activity cells obtained from virus-producing or nonproducing NBC-13 cultures were tested on indicator WI-38 cells (50 × 10^6 NBC-13 cells/75-sq cm Falcon flask). An average of 500 syncytia/flask were observed in the indicator cultures inoculated with the BLV-producing NBC-13 cells whereas only less than 5 syncytia were seen in the cultures inoculated with the nonproducing NBC-13 cells.

Table 5
Comparison of the syncytia-inducing activity of uncultured and cultured BLV-infected BC cells

Influence of incubation time of the indicator cells with the BC cell inoculum.

Incubation time	Inoculum[a]	
	Uncultured BC cells	Cultured BC cells
5 min	2, 1[b]	NT[c]
15 min	8, 7	NT
30 min	15, 20	NT
1 hr	58, 30	>500, >500
3 hr	48, 35	TNTC, TNTC
6 hr	75, 60	TNTC, TNTC
12 hr	202, 131	TNTC, TNTC
24 hr	>500, >500	TNTC, TNTC

[a] One × 10^6 uncultured BC cells or 5 × 10^5 cultured (48 hr) BC cells were added to a Leighton tube containing WI-38 cells seeded 24 hr previously at a cell density of 5 × 10^5 cells/tube. After incubation at 37° for the indicated intervals of time, the BC cells were removed by gently rinsing the tubes twice with Hanks' balanced salt solution. Fresh complete medium (2 ml) was added, and after incubation at 37° for 3 to 4 days, the indicator cells were fixed with methanol and stained with Giemsa.

[b] Number of syncytia in replicate cultures.

[c] NT, not tested; TNTC, too numerous to count.

Syncytia Induction by Isolated BLV. In view of the previous results suggesting that the syncytia-inducing activity of BC cells is due to BLV, experiments were conducted to ascertain whether isolated BLV can induce the CPE. As indicated in Table 6, 4 different monolayer cultures formed syncytia upon inoculation with filtered cell-free BLV filtrates or pellets obtained from 3 different virus-producing cultures. In addition, 2 different preparations of density gradient-purified BLV induced syncytia in the 2 indicator cells tested. The syncytia produced by cell-free preparations of BLV were indistinguishable from those induced by BLV-producing BC cells. The results summarized in Table 7 further illustrate that the numbers of syncytia usually increase upon subcultivation of the indicator cells. The differences in numbers of syncytia in the monolayer cultures inoculated with cell-free preparations of BLV-bat_2 do not necessarily reflect differences in susceptibility of these cultures, since they were not inoculated with the same virus preparations. However, results of other experiments have shown that, of the monolayers tested, BESP cells are the most susceptible to syncytia induction by cell-free preparations of BLV.

Table 6
Syncytia formation in several monolayer cell cultures infected with cell-free preparations of BLV

Viral source	BLV preparation[a]	Indicator monolayer culture[b]	Syncytia formation[c]	
			Before subcultivation	Passage 1
Cell line BLV-bat_2	CFP	WI-38	+	++
		BESP	+++	+++
		S-743	+++	++
		TB1Lu	+	++
	CFF	WI-38	+	++
		BESP	+++	+++
		S-743	+	+
		Tb1Lu	+	+
Cell line BLV-bat_1	CFP	S-743	+	++
		Tb1Lu	+	++
Cell line BLV-bat_2	Density gradient purified	S-743	+	+
		BESP	++	++
Short-term culture of AJ-147 BC cells	CFP	S-743	+	+

[a] CFP, resuspended cell-free BLV pellet; CFE, cell-free filtrate of BLV containing culture fluid (see "Materials and Methods").

[b] Two to 5 ml of the virus preparations were added to a 75-sq cm Falcon flask containing the indicator cells that were pretreated with DEAE-dextran.

[c] Cultures were monitored for syncytia after they reached confluency. Results are expressed as the approximate percentage of the monolayer sheet showing syncytia (+, 1 to 10%; ++, 10 to 25%; +++, >30%).

Table 7
Neutralization of the syncytia-inducing activities of BLV and BLV-infected BC cells by bovine sera

Inoculum incubated with[a]	Syncytia-inducing inoculum: Cell-free BLV filtrate[b, c]	Syncytia-inducing inoculum: AJ-147 BC cells[c]
MEM	>1000	>1000
Sera from normal cattle in leukemia-free Herd BI[d]		
BI-434	>1000	>1000
BI-048	NT[e]	>1000
BI-196	>1000	>1000
Reference BSV sera		
SE-271	>1000	>1000
Se-354	>1000	>1000
Se-344	>1000	>1000
Reference BLV sera		
27-125	0	0
γ-Globulin 27-125	NT	0
Se-276	0	0
γ-Globulin Se-276	0	NT
Se-362	0	0

[a] Two ml of cell-free BLV filtrate were mixed with an equal volume of heat-inactivated undiluted serum or γ-globulin solution (11 mg of protein per ml). The mixture was incubated for 1 hr at 37° and then incubated into a 60-mm Falcon dish containing BESP cells. One ml of MEM containing 1 × 10^6 viable AJ-147 BC cells was mixed with an equal volume of heat-inactivated undiluted serum. The mixture was incubated for 1 hr at 37° and then inoculated into a 60-mm Falcon dish containing WI-38 cells. Each virus-serum and BC cell-serum mixture was tested in duplicate.
[b] From culture medium of cell line BLV-bat_2.
[c] Numbers of syncytia per 60-mm Falcon dish (Integrid).
[d] These sera were negative for BLV antibodies in the IFA test.
[e] NT, not tested.

Inhibition of the Syncytia-inducing Activity of BLV. As shown in Table 7, the syncytia-inducing activity of cell-free preparations of BLV produced by cell line BLV-bat_2 was completely neutralized by sera containing BLV antibodies, but not by serum from normal cows in a leukemia-free herd or by reference BSV sera. Virus-neutralizing antibodies are directed against virion envelope antigens. Thus, the fact that identical results were obtained when the neutralization activity of the sera was tested against BLV-infected BC cells indicates that the BLV particles released by cell line BLV-bat_2 and the bovine BC cells share envelope antigens. Furthermore, in other experiments, we have found that the neutralizing activity of a reference BLV serum for BLV-infected bovine BC cells can be removed completely by absorption with cell-free BLV preparations obtained from cell line BLV-bat_1 or BLV-bat_2. IFA, IFA absorption, and immunodiffusion data have demonstrated that the virus particles produced by BLV-bat_1, BLV-bat_2, and bovine BC cells are antigenically indistinguishable (16, 22, 23).[4] Thus, the immunological data, the fact that the BLV-infected bat cell lines were initiated by cocultivation with BLV-infected cells, and the fact that the viruses present in the bat cell lines and the BC cells have the same *in vitro* biological properties further support the conclusion that the virus produced by the BLV-bat cell lines is indeed BLV.

As an additional control for the specificity of the neutralization tests (Table 7), BSV-infected BC from Cow BH-116 were preincubated with either Se-362 or SE-354 (undiluted or diluted 1:10) or MEM, and inoculated into BESP cells, following the same procedures described above. The indicator cells were monitored for 4 consecutive passages. The BSV-infected BC cells preincubated with either MEM or reference BLV serum Se-362 induced large numbers of syncytia, whereas preincubation with reference BSV serum Se-354, either undiluted or diluted 1:10, completely inhibited the syncytia-inducing activity of the BSV-infected BC cells.

The data in Table 8 clearly demonstrate that UV irradiation, heating for 15 min at 56°, ether treatment, and freezing and thawing destroy the syncytia-inducing activity of BLV.

BLV Antigen Expression and BLV Replication in Syncytia-positive Cultures. WI-38, BESP, Tb1Lu, S-743, and FLS cells were tested by the IFA technique with reference BLV serum Se-276 or 27-125 at various passages after inoculation with either cell-free BLV preparations or AJ-147 BC cells. All of these cultures showed a variable percentage of syncytia and, less frequently, mononucleated cells with intense granular and/or diffuse fluorescence in the cytoplasm. Nuclear staining was not observed. In the cultures inoculated with AJ-147 BC cells and in BESP monolayers infected with cell-free BLV preparations, the fluorescent cells were detected before the cultures were subcultured. On the other hand, in WI-38, Tb1Lu, S-743, and FLS monolayers inoculated with cell-free BLV preparations, fluorescent cells usually began to appear only after 1 or 2 passages, *i.e.*, after the cultures already had syncytial cells. Control uninfected monolayer cell cultures did not react in the IFA test with reference BLV sera.

IFA absorption experiments were conducted to ascertain

Table 8
Inhibition of the syncytia-inducing activity of BLV by various treatments

Virus preparation[a]	Treatment[b]	No. of syncytia/60-mm Falcon dish[c, d]
CFF	None	>1000
	UV irradiation	0
CFF	None	>1000
	Heating (56°, 15 min)	0
	Freezing and thawing	<100
CFP	None	>500
	Freezing and thawing	<50
	Ether-treated, soluble fraction	0
	Ether-treated, precipitable fraction	0

[a] CFF, cell-free filtrate; CFP, cell-free virus pellet, obtained from cell line BLV-bat_2.
[b] See "Materials and Methods."
[c] BESP cells used as indicator. Each untreated or treated virus preparation tested in duplicate.
[d] Integrid.

Table 9
IFA absorption[a] experiment with reference BLV serum 27-125

Antigen used for absorption	Residual IFA activity tested on		
	NBC-13	BLV-infected, syncytia-positive WI-38 cells	BSV-infected BESP cells
PBS	+[b]	+	+
Ether-treated homogenate of BC cells from a cow in leukemia-free herd	+	+	+
Ether-treated homogenate of BSV-infected BESP cells	+	+	–
Ether-treated homogenate of BLV-infected NBC-13 cells	–	–	+
Ether-treated homogenate of BLV-infected BC cultures of Cow AJ-147	–	–	+
Ether-treated homogenate of cells from BLV-bat$_2$	–	–	+
Ether-treated, density gradient purified BLV from BLV-bat$_2$	–	–	+
Ether-treated homogenate of syncytia-positive WI-38 cells[c]	–	–	+

[a] Reference serum 27-125 was diluted 1:100 with an equal volume of the antigen preparation. After incubation the serum was tested for residual fluorescent activity on the indicated target cells.
[b] +, no absorption activity.
[c] The homogenates were prepared with monolayer cells obtained in the 2nd passage after cocultivation with AJ-147 BC cells.

further the presence of BLV antigen in the syncytia-positive cultures. The results (Table 9) show that ether-treated homogenates of BLV-infected syncytia-positive WI-38 cells, BLV-infected NBC-13 cells, and BLV-bat$_2$ cells, as well as ether-treated BLV from the BLV-bat$_2$ culture, completely removed the fluorescent antibodies of serum 27-125 reacting with NBC-13 cells or BLV-infected WI-38 cells, but not the antibodies reacting with BSV-infected BS-2 target cells. On the other hand, the fluorescent activity of serum 27-125 for BS-2 cells was removed by absorption with homogenates of these cells, but not by absorption with any of the homogenates of BLV-infected cells or the ether-treated virus. From these results it is clear that the syncytia-positive cell cultures inoculated with BLV contain the antigen(s) of this virus but not the antigen(s) of BSV.

Syncytia-positive cultures showing fluorescent reactions with reference BLV sera were also repeatedly tested with reference sera for BSV, R-29, IBR, PI$_3$, and BVD viruses. In all cases the results were negative.

The RDDP assay and, in several cases, electron microscopy were applied to detect the presence of complete BLV in the syncytia-positive infected cultures. WI-38 and BESP monolayers occasionally showed very low levels of enzyme activity and a few C-type virus particles in the 1st 2 passages. Subsequently, however, these cultures became completely negative for BLV despite the presence of extensive CPE and fluorescent cells. The other syncytia-positive infected monolayers began to produce BLV in early passages when inoculated with BLV-infected BC cells or after several passages when inoculated with cell-free BLV preparations. As mentioned above, these cultures showed fluorescent cells either before subcultivation or after 1 or 2 passages. Virus production increased with passage and was consistently abundant in some of the Tb1Lu cultures. These are the cultures designated as BLV-bat$_1$ and BLV-bat$_2$ from which some of the cell-free preparations of BLV used in the experiments reported in Tables 6 to 8 were obtained. In the syncytia-positive S-743 and FLS cultures, BLV production fluctuated and sometimes ceased completely. A study of the propagation of BLV in various cell cultures will be reported elsewhere.

Table 10 summarizes the results of monolayers that were tested for the presence of syncytia, BLV, and BLV antigen in the same passages. The data clearly show that the extent of syncytia involvement did not necessarily correlate with the degree of virus production and virus antigen expression in the indicator cell cultures.

DISCUSSION

These data have established that either normal or leukemic lymphocytes infected with BLV induce syncytia in WI-38 cells as well as in monolayer cell cultures of bovine, simian, ovine, caprine, and bat origin. In contrast, the mouse and rat cells tested were refractory to this CPE. That the syncytia effect induced by infected bovine lymphocytes is specific is evident from the fact that syncytia were not induced in WI-38 cells by bovine lymphocytes uninfected with BLV.

The BLV-infected cells need not be viable to induce syncytia, since WI-38 cells inoculated with cell-free homogenates of BLV-producing cells also showed the CPE.

Table 10
Syncytia formation, virus production and virus antigen expression in monolayer cell cultures inoculated with BLV or BLV-producing BC cells

Indicator cells	Cell passage	Syncytia formation[a]	RDDP assay[b]	IFA[c] (%)
WI-38	1	++	0	5
BESP	1	+++	1,314	10
BESP	2	+++	304	2
S-743	6	+	25,733	40
Tb1Lu	28[d]	++	57,030	35
Tb1Lu	46[d]	+	53,329	20
Tb1Lu	9[e]	+++	12,140	5
Tb1Lu	42[e]	++	3,409	20

[a] Results are expressed as the approximate percentage of the monolayer sheet showing syncytia (+, 1 to 10%; ++, 10 to 25%; +++, >30%).
[b] Results expressed as the cpm of [^{3}H]TMP incorporated into poly(dT) product during incubation for 60 min at 37°. Values were corrected by subtracting 3 times the cpm of the blank.
[c] The percentage of positive cells in IFA test with reference BLV serum was determined by examining at least 200 cells.
[d] Cell line BLV-bat$_2$.
[e] Cell line BLV-bat$_1$.

As in the case of BC cells from all BLV-infected cattle studied in our laboratory (10, 33), uncultured AJ-147 BC cells do not release detectable virus particles. However, BLV production occurs soon after the infected cells are grown *in vitro* and increases thereafter. Thus, our observation that the longer the incubation period of monolayer and AJ-147 BC cells, the stronger the syncytia response, may be interpreted to indicate that BLV itself is responsible for the CPE. This view is supported further by the following findings: *(a)* cultured AJ-147 BC cells induced higher numbers of syncytia than a double inoculum of uncultured cells of the same stock; *(b)* cell-free homogenates of cultured AJ-147 BC cells induced syncytia, whereas homogenates of uncultured AJ-147 BC cells did not; *(c)* samples of BLV-producing cultures of cell line NBC-13 had syncytia-inducing activity, whereas samples of nonproducing NBC-13 cultures were virtually negative; and *(d)* more conclusively, cell-free preparations of BLV obtained from either AJ-147 BC cell cultures or the BLV-bat cell lines, as well as density gradient-purified virus, induced syncytia. However, these findings do not rule out the possibility that, in addition to BLV, a cell factor synthesized during BLV replication may also participate in the syncytia-inducing activity of the BC cells.

The possibility that the CPE observed in this study was due to BSV, R-29, PI_3, IBR, or BVD viruses rather than to BLV can be ruled out by the following findings. *(a)* Careful and periodic investigations have failed to detect these viruses in AJ-147 BC cells, cell line NBC-13, or in the monolayer cell cultures (BLV-bat_1 and BLV-bat_2) from which syncytia-inducing virus preparations were obtained. *(b)* WI-38 cells are refractory to the syncytia-inducing activity of BSV-infected BC cells and IBR virus. In addition, when these cells were infected with BVD virus, degenerative changes, but not syncytia formation, were noted. *(c)* Neither BSV, R-29, PI_3, IBR, nor BVD viruses could be found in syncytia-positive indicator cultures inoculated with either BLV-producing AJ-147 BC cells or isolated BLV. *(d)* While reference BLV bovine sera inhibit the syncytia-inducing activity of BLV, reference BSV sera do not.

The ability to induce syncytia in monolayer cultures of apparently normal cells further differentiates BLV from the other mammalian C-type leukemia viruses. None of these agents has been shown to induce syncytia except in cells transformed by and carrying the RSV genome (XC and KC cell) (18, 29, 30, 32). The only other C-type virus known to induce syncytia in normal cells is the endogenous feline A262 virus. It is not known whether this virus has oncogenic properties (26). None of the cell cultures susceptible to the CPE of BLV show signs of transformation, and there is no evidence that they carry the RSV genome. In addition, XC cells cocultivated with BLV-producing BC cells do not form syncytia. Previous work has shown that BLV is not only antigenically different from other mammalian leukemia viruses, but also lacks the interspecies-specific antigenic determinants shared by these agents (7, 10, 22). It has also been reported that BLV is morphologically somewhat different from the other C-type viruses (3, 36). Thus, on the basis of the above information, it seems clear that BLV is not a typical C-type virus and, as proposed earlier (6), may be the prototype of a new family of leukemic viruses.

The observation by Cornefert-Jensen *et al.* (4) of syncytia formation in WI-38 cells and 2 other monolayer cultures cocultivated with bovine leukemic cells appears to represent the same phenomenon described here. Although these authors reported their findings before BLV was identified, the presence of the virus in the leukemic cell inocula used can be assumed. Indeed, subsequent studies have shown that the BC cells of most leukemic cows are infected with BLV (10, 24, 33). More recently, Van der Maaten *et al.* (36) also noted syncytia formation in BLV-releasing monolayer cell cultures and speculated on the possibility that the CPE was due to this virus.

The mechanism of syncytia formation by BLV or BLV-infected cells has not been investigated. However, the rapidity with which the CPE occurs and the absence of mitotic figures in the syncytia suggest that the CPE arises by fusion of the cells rather than by endomitosis.

The fusion factor does not seem to be the ether-resistant BLV internal antigen (7, 9, 22) since ether-treated BLV does not induce syncytia. The finding that UV irradiation, as well as freezing and thawing, destroys the syncytia-inducing activity of BLV argues against the possibility that the fusion factor is another component of the BLV particle. The latter observations and the fact that incubation with reference BLV sera specifically prevents the syncytia-inducing activity of BLV indicate that this activity requires infection of the indicator cells. That these cells do become infected is supported by the observation that they consistently show BLV antigen in IFA tests. On the other hand, our data indicate that the syncytia response to BLV is independent of whether or not the virus is produced by the indicator cells. BLV-infected WI-38 and BESP cultures, despite extensive CPE, do not usually produce BLV; the few virus particles and low levels of RDDP detected occasionally in the 1st 2 passages of these cultures probably represent the virus present in the inoculum rather than virus produced by the indicator cells. Moreover, in S-743 cells and particularly in Tb1Lu cells, BLV production may be very high, whereas syncytia formation may be only moderate or low. Thus, syncytia formation cannot be used as an indicator of BLV production in monolayer cell cultures.

Studies reported in a companion paper (13) are concerned with the development of a sensitive, rapid, and practical infectivity assay for BLV on the basis of its syncytia-inducing activity. This assay also provides a means for detecting and titrating BLV-neutralizing antibodies.

The fact that human WI-38 cells are susceptible to infection with BLV deserves particular attention because of the possibility that BLV represents a potential hazard for humans.

ACKNOWLEDGMENTS

We are indebted to Dr. Robert R. Marshak for his help. We also thank Dr. E. M. Levine for testing our cultures for *Mycoplasma* contamination and Dr. D. C. Graves for supervising the RNA-dependent DNA polymerase assays.

REFERENCES

1. Abt, D. A., and Marshak, R. R. Bovine Leukemia. *In:* M. A. Rich (ed.), Experimental Leukemia, pp. 191–204. New York: Appleton-Century-Crofts, 1968.

2. Abt, D. A., Marshak, R. R., Kulp, H. W., and Pollock, R. J. Studies on the Relationship between Lymphocytosis and Bovine Leukosis. Bibliotheca Haematol., *36:* 527–536, 1970.
3. Calafat, J., Hageman, P. C., and Ressang, A. A. Structure of C-type Virus Particles in Lymphocyte Cultures of Bovine Origin. J. Natl. Cancer Inst., *52:* 1251–1257, 1974.
4. Cornefert-Jensen, F., Hare, W. C., and Stock, N. D. Studies on Bovine Lymphosarcoma: Formation of Syncytia and Detection of Virus Particles in Mixed Cell Cultures. Intern. J. Cancer, *4:* 507–519, 1969.
5. Cornefert-Jensen, F., Stock, N. D., and Marshak, R. R. Detection of a Bovine Virus Associated with Syncytial Formation in Mixed Cell Cultures. *In:* R. M. Dutcher (ed.), Comparative Leukemia Research. Vol. 36, pp. 453–464. Basel: S. Karger AG, 1970.
6. Diglio, C. A., Hare, W. C. D., Dodd, D. C., Marshak, R. R., and Ferrer, J. F. Cytogenetic, Cytological and Virological Characteristics of a Bovine Fibrosarcoma. Cancer Res., *35:* 3628–3635, 1975.
7. Ferrer, J. F. Antigenic Comparison of Bovine Type C Virus with Murine and Feline Leukemia Viruses. Cancer Res., *32:* 1871–1877, 1972.
8. Ferrer, J. F., Abt, D. A., Bhatt, D. M., and Marshak, R. R. Studies on the Relationship between Infection with Bovine C-type Virus, Leukemia and Persistent Lymphocytosis in Cattle. Cancer Res., *34:* 893–900, 1974.
9. Ferrer, J. F., Avila, L., and Stock, N. D. Serological Detection of Type C Viruses Found in Bovine Cultures. Cancer Res., *32:* 1864–1870, 1972.
10. Ferrer, J. F., Avila, L., and Stock, N. D. Recent Electron Microscopic and Immunologic Studies on Bovine Cell Cultures Containing C-type Viruses. Bibliotheca Haematol., *39:* 206–214, 1973.
11. Ferrer, J. F., and Bhatt, D. M. Occurrence of Fluorescent and Precipitin Antibodies to a Bovine C-type Virus (BLV) among the Cattle Population. Proc. Am. Assoc. Cancer Res., *14:* 118, 1973.
12. Ferrer, J. F., Bhatt, D. M., Abt, D. A., Marshak, R. R., and Baliga, V. Serological Diagnosis of Infection with the Putative Bovine Leukemia Virus. Cornell Vet., *65:* 527–542, 1975.
13. Ferrer, J. F., and Diglio, C. A. Development of an *in Vitro* Infectivity Assay for the C-type Bovine Leukemia Virus. Cancer Res., *36:* 1068–1073, 1976.
14. Ferrer, J. F., Guilleman, B., and Stock, N. D. Tissue Culture Condition Affecting the Expression of Latent C-type Viruses in Leukemia. XIVth International Congress of Hematology, San Paulo, Brazil, 1972.
15. Ferrer, J. F., Stock, N. D., and Lin, P. S. Detection of Replicating C-type Viruses in Continuous Cell Cultures Established from Cows with Leukemia: Effect of the Culture Medium. J. Natl. Cancer Inst., *27:* 613–621, 1971.
16. Graves, D. C., Piper, C. E., and Ferrer, J. F. Preliminary Biochemical and Biophysical Characterization of the Bovine C-type Virus (BLV). Abstract. *In:* Annual Meeting of the American Society for Microbiology, p. 261, 1975.
17. Hare, W. C. D., Lin, P. S., and Zachariasewycz, E. Studies on 12 Cell Lines Established from Mononuclear Leukocytes of Cattle with Lymphosarcoma. Cancer, *22:* 1074–1084, 1968.
18. Klement, V., Rowe, W. P., Hartley, J. W., and Pugh, W. E. Mixed Culture Cytopathogenicity: A New Test for Growth of Murine Leukemia Virus in Tissue Culture. Proc. Natl. Acad. Sci. U. S., *63:* 753–758, 1969.
19. Malmquist, W. A., Van der Maaten, M. J., and Boothe, A. D. Isolation Immunodiffusion, Immunofluorescence and Electron Microscopy of a Syncytial Virus of Lymphosarcomatous and Apparently Normal Cattle. Cancer Res., *29:* 188–200, 1969.
20. Marshak, R. R. Criteria for the Determination of the Normal and Leukotic State in Cattle. J. Natl. Cancer Inst., *41:* 243–263, 1968.
21. Marshak, R. R., Hare, W. C. D., Abt, D. A., Croshaw, J. E., Jr., Switzer, J. W., Ispen, J., Dutcher, R. M., and Martin, J. E. Occurrence of Lymphocytosis in Dairy Cattle Herds with High Incidence of Lymphosarcoma. Ann. N. Y. Acad. Sci., *108:* 1284–1301, 1963.
22. McDonald, H. C., Graves, D. C., and Ferrer, J. F. Characterization of Internal Antigen of the Bovine C-type Virus. Proc. Am. Assoc. Cancer Res., *15:* 95, 1974.
23. McDonald, H. C., Graves, D. C., and Ferrer, J. F. Isolation and Characterization of an Antigen of the Bovine C-type Virus. Cancer Res., *36:* 1251–1257, 1976.
24. Miller, J. M., Miller, L. D., Olson, C., and Gillette, K. G. Virus-like Particles in Phytohemagglutinin-Stimulated Lymphocyte Cultures with Reference to Bovine Lymphosarcoma. J. Natl. Cancer Inst., *43:* 1297–1305, 1969.
25. Miller, J. M., and Olson, C. Precipitating Antibody to an Internal Antigen of the C-type Virus Associated with Bovine Lymphosarcoma. J. Natl. Cancer Inst., *49:* 1450–1462, 1972.
26. Noronha, F., Dougherty, E., Poco, A., Gries, C., Post, J., and Rickard, C. Cytological and Serological Studies of a Feline Endogenous C-type Virus. Arch. Ges. Virusforsch., *45:* 235–248, 1974.
27. Olson, C., Hoss, H. E., Miller, J. M., and Baumgartener, L. E. Evidence of Bovine C-type (Leukemia) Virus in Dairy Cattle. J. Am. Vet. Med. Assoc., *163:* 355–357, 1973.
28. Olson, C., Miller, L. D., Miller, J. M., and Hoss, H. E. Transmission of Lymphosarcoma from Cattle to Sheep. J. Natl. Cancer Inst., *49:* 1463–1467, 1972.
29. Rand, K. H., and Long, C. Syncytial Assay for the Putative Human C-type Virus, RD-114, Utilizing Human Cells Transformed by Rous Sarcoma Virus. Nature New Biol., *240:* 187–190, 1972.
30. Rangan, S. R. S., Wong, M. C., Moyer, P. P., and Jensen, E. M. Cytopathogenic Test for Feline Leukemia Virus Infections. Appl. Microbiol., *23:* 628–636, 1972.
31. Ross, J., Scolnick, E. M., Todaro, G. J., and Aaronson, S. A. Separation of Murine Cellular and Murine Leukemia Virus DNA Polymerases. Nature New Biol., *231:* 163–167, 1971.
32. Rowe, W. P., Pugh, W. E., and Hartley, J. W. Plaque Assay Techniques for Murine Leukemia Viruses. Virology, *42:* 1136–1139, 1970.
33. Stock, N. D., and Ferrer, J. F. Replicating C-type Virus in Phytohemagglutinin-treated Buffy Coat Cultures of Bovine Origin. J. Natl. Cancer Inst., *48:* 985–996, 1972.
34. Todaro, G. J., Benveniste, R. E., Lieber, M. M., and Sherr, C. J. Characterization of a Type C Virus Released Fom the Porcine Cell Line PK(15). Virology, *58:* 65–74, 1974.
35. Van der Maaten, M. J., Boothe, A. D., and Seger, C. L. Isolation of a Virus from Cattle with Persistent Lymphocytosis. J. Natl. Cancer Inst., *49:* 1649–1675, 1972.
36. Van der Maaten, M. J., Miller, J. M., and Boothe, A. D. Replicating Type C Virus Particles in Monolayer Cell Cultures of Tissues from Cattle with Lymphosarcoma. J. Natl. Cancer Inst., *52:* 491–497, 1974.

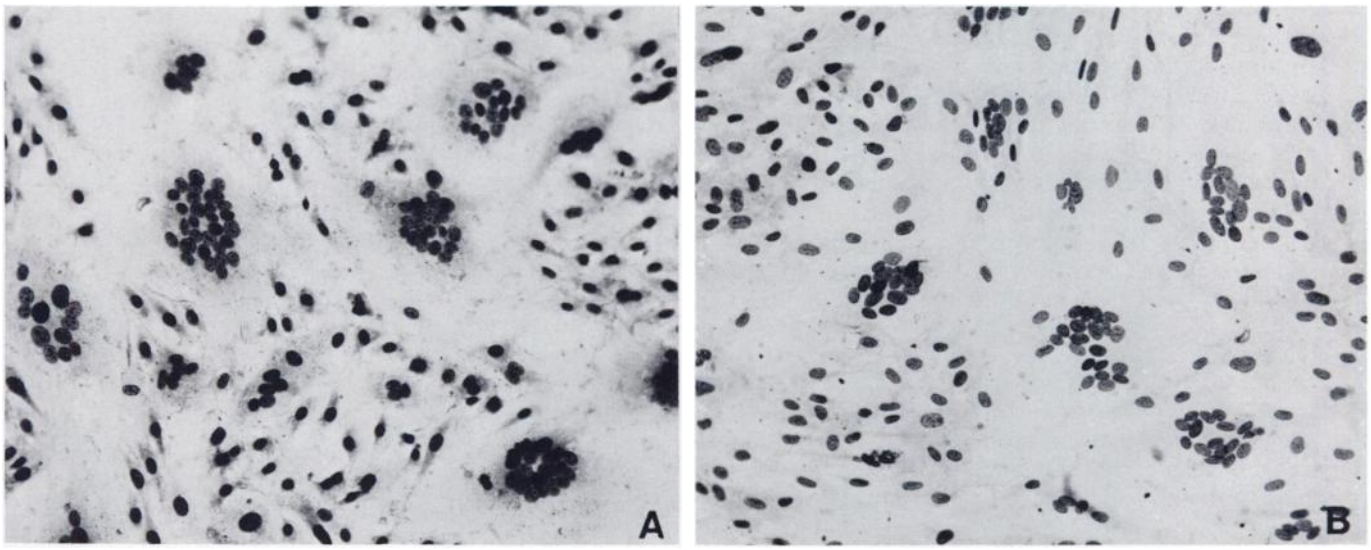

Fig. 1. *A*, typical syncytia induced by BLV on BESP cells. × 125. *B*, typical syncytia induced by BLV on WI-38 cells. × 125. Cells were washed with PBS, fixed with methanol, and stained with Giemsa.