Establishment and Propagation of a Bovine Leukaemia Virus-producing Cell Line Derived from the Leukocytes of a Leukaemic Cow

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SUMMARY

A cell line (LB59Ly) derived from the leukocytes of a leukaemic cow was established as a monolayer, which spontaneously released large amounts of a retrovirus. This virus was found to be indistinguishable from the bovine leukaemia virus (BLV) produced by the commonly used high-producing heterologous cell line (FLK-BLV). Like the latter, its reverse transcriptase activity was greater in the presence of Mg$^{2+}$ cations than in the presence of Mn$^{2+}$ cations; its polyacrylamide gel electrophoresis pattern showed the presence of gp51, p24, p15, p12 and p10, and the antigenicity of the two major proteins completely cross-reacted with those of BLV from FLK-BLV cells. The virus was infectious and induced early and late polykaryocytosis, the specificity of which was demonstrated by use of specific anti-BLV sera.

INTRODUCTION

Bovine leukaemia virus (BLV), a member of the retroviridae family, has been shown to be the causative agent of the enzootic form of bovine lymphosarcoma (Miller et al., 1969; Kawakami et al., 1970; Kettmann et al., 1978). However, this virus has not been found at a detectable level in infected animals. Therefore, it appeared necessary to try to develop an abundant in vitro source of BLV for virological, biochemical and immunological studies. For several years efforts have been made in vain in different laboratories to obtain permanent in vitro cell lines producing large amounts of BLV. Although it was relatively easy to establish cell lines of lymphoid origin from leukaemic cattle (Hare et al., 1968; Ressang et al., 1974), the cells produced either no or very limited amounts of virus-like particles. We have shown that, in short-term and long-term cultures of leukaemic leukocytes, BLV production is associated with cell degeneration and death (Guillemain et al., 1976). Such a situation has hampered fundamental studies on BLV and its relationships with the disease.

A number of authors have investigated the permissiveness of heterologous cells. Infection with BLV was performed either directly or by co-cultivation. The best results were obtained by Van Der Maaten & Miller (1976) who succeeded in infecting foetal lamb kidney cells (FLK) with in vitro-derived BLV. The FLK-BLV cell line was readily shown to maintain its virus production capacity. This tool, generously supplied to the scientific community, has contributed greatly to the recent and rapid development in our knowledge of bovine leukaemia and related viruses.

In view of the fact that in cattle natural BLV infection is restricted to lymphocytes (Paul et al., 1977; Kenyon & Piper, 1977; Kettmann et al., 1978), we have investigated the possibility of obtaining in vitro established cell lines of lymphoid origin from cattle with lymphosarcoma which spontaneously release BLV or from normal cattle.

One such cell line termed LB59Ly is the object of this report. We have shown that
monolayers can be easily derived from bovine leukaemic lymphoid cells and can be maintained permanently without any cell degeneration; such cells produce large amounts of virus particles. These particles cannot be distinguished from BLV produced by FLK-BLV cells on the basis of biophysical, biochemical or antigenic properties. Furthermore, we have shown that the virus released by LB59Ly cells is infectious for homologous cells and particularly for in vitro-derived bovine normal lymphoid cells.

**METHODS**

**Animal.** LB59 was a 5 year-old Francaise Frisonne Pie Noire cow bearing a multicentric lymphosarcoma. Tumourous materials were kindly provided by Dr A. L. Parodi (Ecole Vétérinaire d'Alfort, Maisons-Alfort, France). The blood picture was characteristic of lymphocytosis according to the E.E.C. key.

**Cell cultures.** Bovine lymphoid blood cells from LB59 (LB59Ly cells) were obtained by the improved ammonium chloride solution method of Odajima & Sonoda (1971). The collected cells were then seeded and maintained as monolayers in RPMI 1629 medium supplemented with 20% heat-inactivated (56 °C, 10 min) foetal calf serum (FCSI), 4 ng/ml β-mercaptoethanol, 100 units/ml penicillin and 100 µg/ml streptomycin. Once established, the culture was maintained as described below for the other cell lines. The LyNX cell line was derived and established from the leukocytes of a normal cow free of BLV infection. The MOL cell line was derived and established from the femoral marrow of a 4 month-old bovine foetus. FLK cells chronically infected with the bovine leukaemia virus (FLK-BLV) were kindly supplied by Drs M. J. Van der Maaten and J. M. Miller (National Animal Disease Laboratory, U.S. Department of Agriculture, Ames, Iowa, U.S.A.). Bovine embryo spleen cell (BESP) cultures were developed according to Malmquist et al. (1969) using the spleen of a 4-month-old foetus kindly supplied by Dr E. Leftheriotis (Institut Merieux, Marcy-l'Etoile, France). The murine sarcoma virus-infected cat cell line 8C81, a subclone of the CC81 cell line (Fischinger et al., 1974) was kindly supplied by Dr J. C. Chermann (Institut Pasteur, Paris, France). The 13-3C is a cell line chronically producing a murine radiation leukaemia virus (RadLV-Rs) (Astier et al., 1976).

All these cell lines were maintained as monolayers in RPMI 1629 culture medium supplemented with 10% FCSI, 100 units/ml penicillin and 100 µg/ml streptomycin.

**Virus concentration and purification.** The viruses were concentrated and gradient purified from the culture medium of FLK-BLV, LB59Ly and 13-3C cells as reported previously (Astier et al., 1976). Labelled virus was obtained from cultured cells incubated for 24 h in medium containing 3H-uridine (20 µCi/ml).

**Viral antigens from BLV released by FLK-BLV cells.** The gp51 antigen was purified by gel filtration in guanidine-HCl as described by Fleissner (1971). BLV gp envelope antigen (Seromed, Munich, F.R.G.) used in immunodiffusion experiments was kindly supplied by Biopro, Lille, France; no p24 antigen was detectable (in batch no. 977-26) by the polyacrylamide gel electrophoresis (PAGE) technique described below. The p24 antigen was prepared as described by Devare et al. (1976).

**Sera.** LB44 and LB71 sera were from lymphosarcomatous cows naturally infected with BLV and contained early polykaryocytosis (EP)-inhibiting antibodies (see below) with respective titres of 532 and 1596 SID25N units/0-1 ml. LB59 serum will be described in Results. Rabbit sera were obtained after immunization with Triton X-100-disrupted BLV or with BLV-purified p24. The S reference serum from Seromed used in immunodiffusion experiments, preferentially directed against BLV gp envelope antigen, was kindly supplied by Biopro.

**Immunodiffusion (ID) test.** This test was performed using Hyland immunoplates. Total BLV antigens were used after disruption of the purified virus in the presence of 0.1% Triton
Bovine lymphoid leukaemic cells producing BLV

X-100. BLV gp envelope antigen was from Seromed and BLV p24 antigen was prepared in our laboratory.

**Competitive radioimmunoassay.** The p24 antigen was iodinated (125I) by the chloramine-T method (Greenwood *et al.*, 1963) and the gp51 was iodinated by the iodogen method (Devare & Stephenson, 1977).

Bovine leukaemia viruses released either by FLK-BLV cells or LB59Ly cells, murine leukaemia virus (MuLV) produced by 13-3C cells and BLV p24 and BLV gp51 antigens were used. Serial twofold dilutions of the unlabelled competing antigen were incubated with the rabbit anti-BLV serum at 37 °C for 1 h in 0.2 ml reaction mixture containing 0·01 M-tris-HCl pH 7·9; 0·05 M-NaCl; 1 mM-EDTA; 0·1% Triton X-100; 1% bovine serum albumin. 125I-labelled protein (20000 ct/min) was then added and incubated at 37 °C for 3 h and overnight at 4 °C. A 10% suspension of *Staphylococcus aureus* prepared according to Kessler (1975) was added and the whole mixture was further incubated for 30 min at 4 °C. To this, 500 µl 0·01 M-tris–HCl pH 7·9; 0·1 M-NaCl; 1 mM-EDTA; 0·1% Triton X-100 buffer was added. The mixture was then centrifuged (3600 g, 15 min) and the resulting pellet washed and recentrifuged in the same conditions in 0·5 ml of the above buffer. The radioactivity of the pellet was then determined.

**Polyacrylamide gel electrophoresis (PAGE).** The protein samples were heated at 100 °C for 2 min in 25 mM-tris–HCl pH 7·9; 0·1 M-β-mercaptoethanol; 1% SDS. The preparations were then adjusted to 10% glycerol, layered on the top of slab polyacrylamide gel gradients (7·5 to 17·5%) and the separation of proteins was achieved according to Laemmli (1970).

**Reverse transcriptase assay.** Viruses (30 µg) pretreated with 0·05% Triton X-100 for 10 min at 4 °C were incubated for 60 min at 37 °C in a final vol. of 50 µl of the following reaction mixture: 50 mM-tris–HCl pH 8·3; 40 mM-NaCl; 20 mM-MgCl2; 15 mM-dithiothreitol; 10−3 mM-3H-TTP; 1 mM-dATP, -dCTP, -dGTP. The acid-insoluble radioactivity was then determined. Polymerase I from *Escherichia coli* used as control was commercially obtained (Boehringer, Mannheim) and tested with 'activated' calf thymus DNA.

**Early polykaryocytosis-inhibition (EPI) test.** This test was conducted as described previously (Guillemain *et al.*, 1978). In brief, the test was performed in microtest plates for tissue culture, 5 × 10^3 FLK-BLV cells/well were incubated with serial dilutions (0·1 ml) of the serum to be tested (1 h, 37 °C), 2 × 10^4 8C81 cells were then added and the co-cultures further incubated for 24 h (37 °C). After fixation with methanol and staining with Giemsa, the 50% syncytial inhibition was estimated and normalized (SID50N units).

**Early syncytial induction and neutralization tests.** Lymphoid cells to be tested for early syncytial induction were used either untreated or after incubation with serial dilutions of heat-inactivated bovine sera. Early syncytial induction on BESP or 8C81 cells with the above mentioned cells was performed either in microtest plates for tissue culture in the conditions of the EPI test or in 25 cm^2 tissue culture flasks co-seeded with 2·5 × 10^6 indicator cells and serial densities of the cells to be tested. After incubation for 24 h at 37 °C in 5 ml culture medium the cell layer was fixed, stained with Giemsa and the total number of syncytia scored.

**Virus-induced late syncytial induction.** The presence of syncytia induced by infectious virus in the culture medium of LB59Ly or FLK-BLV cells was determined after infection of BESP, LyNX or MOL cells according to Van der Maaten & Miller (1977) with slight modifications. The presence of BLV-induced syncytia was recorded 6 days after infection by examination of fixed and stained (Giemsa) monolayers.

**RESULTS**

**Serological status of cow LB59**

Tests conducted on the serum of cow LB59 revealed the presence of high levels of EP-inhibiting antibodies, BLV-neutralizing antibodies and BLV-precipitating antibodies
Table 1. Results of serological tests performed on LB59 serum

<table>
<thead>
<tr>
<th>EPI*</th>
<th>VNT†</th>
<th>RIA‡</th>
<th>ID§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1060</td>
<td>&gt; 6000</td>
<td>6000</td>
<td>1000</td>
</tr>
</tbody>
</table>

* EPI, Early polykaryocytosis-inhibition test (SID_{50}N units).
† VNT, Virus neutralization test (50% SI units).
‡ RIA, Radioimmunological assay for BLV p24 or BLV gp51 (dilution precipitating 50% of labelled antigen).
§ ID, Immunodiffusion test (+ indicates presence of a line of precipitation).

detectable by immunodiffusion. Radioimmunoassays aimed at the detection of anti-BLV gp51 and anti-BLV p24 antibodies revealed the presence in the serum of high concentrations of both types of antibodies. These results, which indicate that cow LB59 was heavily infected with BLV, are summarized in Table 1.

Derivation of a monolayer cell line from the leukocytes of cow LB59

The leukocytes harvested from the blood of cow LB59 were seeded at a concentration of 2 × 10^8 to 5 × 10^8 cells per 75 cm² plastic tissue culture flask. The culture medium was supplemented with 20% FCSř negative in the EPI test and 4 ng/ml β-mercaptoethanol as a non-specific mitogen for resting haematopoietic cells. After 24 h incubation, the upper half of the culture medium was carefully replaced. In less than 3 days numerous cells were attached to and flattened on the plastic support. Thereafter, the culture medium was replaced twice a week. Within 5 to 7 days after initiation of the culture there was evident cell proliferation and a confluent monolayer was achieved 2 weeks later. At this stage the cells were maintained by subculturing twice a week at a split ratio of 1:2 for a month and, thereafter, at ratios increasing progressively up to 1:10.

The first confluent cell monolayer and early passages were characterized by a mixture of different morphological cell types. The monolayer itself was composed mainly of fibrocyte-like cells and numerous macrophage-like cells; numerous clusters of round cells were also present on the monolayer. A large number of giant syncytia were also observed. These syncytia progressively disappeared with further passages and the morphology of the cultures tended to become homogeneous and only large epithelial-like cells were present. After establishment of a continuous culture (30 passages), contact inhibition decreased and high densities of long spindle-shaped cells were regularly obtained. Once established, the cell line was termed LB59Ly to indicate its lymphoid origin.

Physicochemical characterization of the virus produced by LB59Ly cells

To test for the presence of BLV production by LB59Ly cells, the following experiments were carried out. Equilibrium density gradients performed after a purification-type procedure for virus produced in medium supplemented with ³H-uridine revealed the presence of RNA-containing particles migrating at a density of 1.16 g/ml, a density characteristic for retroviruses.

Like FLK-BLV, virus gradient-purified LB59Ly virus was also shown to contain a DNA polymerase endogenous activity (Table 2). The high resistance of this activity to actinomycin D is characteristic of RNA-dependent DNA polymerases (reverse transcriptase enzyme). In addition, for the two virus isolates the activity was shown to be 5 to 10 times greater in the presence of Mg^2+ cations than in the presence of Mn^2+ cations.

Virus production by LB59Ly cells was found to be high, as reflected by the content of virus after gradient purification. Depending on the culture conditions, between 2 mg and 7 mg virus/l of culture medium were obtained, a quantity which compares well with the production by FLK-BLV cells.
**Table 2. Properties of the reverse transcriptase enzyme activity of LB59Ly virus**

<table>
<thead>
<tr>
<th>DNA polymerase origin</th>
<th>Reaction mixture</th>
<th>=[H] TMP incorporated into acid-insoluble material (ct/min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLK-BLV virus</td>
<td>Complete</td>
<td>4201</td>
</tr>
<tr>
<td></td>
<td>Complete + Act. D</td>
<td>1748 (42)†</td>
</tr>
<tr>
<td>LB59Ly virus</td>
<td>Complete</td>
<td>933</td>
</tr>
<tr>
<td></td>
<td>Complete + Act. D</td>
<td>446 (48)</td>
</tr>
<tr>
<td>E. coli†</td>
<td>Complete</td>
<td>114 805</td>
</tr>
<tr>
<td></td>
<td>Complete + Act. D</td>
<td>17 118 (15)</td>
</tr>
<tr>
<td>FLK-BLV virus</td>
<td>Complete -Mg$^{2+}$ + 0.5 mM-Mn$^{2+}$</td>
<td>10 366</td>
</tr>
<tr>
<td></td>
<td>Complete -Mg$^{2+}$ + 0.5 mM-Mn$^{2+}$</td>
<td>11 118 (15)</td>
</tr>
<tr>
<td>LB59Ly virus</td>
<td>Complete -Mg$^{2+}$ + 0.5 mM-Mn$^{2+}$</td>
<td>5 096</td>
</tr>
<tr>
<td></td>
<td>Complete -Mg$^{2+}$ + 0.5 mM-Mn$^{2+}$</td>
<td>10 79</td>
</tr>
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* For the actinomycin D (Act. D) inhibition experiments (100 µg/ml) the reaction was carried out at 37 °C for 30 min.
† Percentage of residual activity.
‡ E. coli polymerase I.

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**Fig. 1.** Polyacrylamide gel analysis of (a) MuLV from 13-3C cells, (b) BLV from FLK-BLV cells and (c) BLV from LB59Ly cells. The mol. wt. (x 10^-3) of the bands were deduced from the positions of marker proteins: α-phosphorylase, 94K; BSA, 68K; ovalbumin, 44K; and cytochrome c, 12.5K.

The virus released by LB59Ly cells was further analysed by the PAGE technique. Fig. 1 shows that the pattern of protein distribution, namely gp51, p24, p15, p12 and p10 is identical to that of BLV released by FLK-BLV cells.

**Antigenic characterization of the virus produced by LB59Ly**

The antigenicity of the two major viral proteins of LB59Ly virus (p24 and gp51) was compared to that of FLK-BLV virus in ID tests using Triton X-100-disrupted virions.

Using an anti-BLV gp51 spontaneous bovine serum (S), the identity lines obtained revealed...
identical antigenicities for the glycoproteins of the two viruses. In the same manner, a complete identity of p24 was observed in the two disrupted viruses using a specific rabbit anti-BLV p24 serum.

The complete identity of the antigenicity of the two major antigens was confirmed in competitive radioimmunoprecipitation assays. As shown in a typical experiment presented in Fig. 2, the proteins contained in both viruses competed equally well with a purified BLV gp51 (Fig. 2a) or a purified BLV p24 (Fig. 2b). In contrast, no reaction occurred in the same conditions with MuLV preparations.

**Syncytial induction by LB59 cells**

In a first set of experiments lymphocytes harvested from the blood of cow LB59 were cultured for 72 h. The surviving cells were then co-seeded with BESP cells. After 24 h incubation the monolayers were fixed and stained and the number of syncytia was determined. These syncytia contained at least 6 nuclei/cell and their morphology was similar to that obtained in co-cultures of FLK-BLV and BESP cells. However, unlike FLK-BLV cells, only a fraction of the short-term cultured lymphocytes could be recorded as syncytia-inducing centres ($2 \times 10^{-3}$ SIU/cell).

In a second set of experiments (Fig. 3) long-term cultured LB59Ly cells were assayed for their syncytia-inducing activity in co-cultures with 8C81 cells. Early polykaryocytosis was
Bovine lymphoid leukaemic cells producing BLV

Fig. 3. Regression analysis of the number of syncytia-inducing LB59Ly cells versus the number of syncytia obtained in mixed culture with 8C81 cells. Y represents the number of syncytia-inducing units (SIU) obtained per X LB59Ly cells. X1 denotes the maximum amount of LB59Ly cells seeded and Xd that amount at dilution d.

routinely observed and syncytia appeared as early as 1 h after co-cultivation and their maximum quantity was consistently observed after 24 h incubation. The size, morphology and nuclei content of these giant cells (often containing more than 100 nuclei) were similar to those induced by FLK-BLV cells. Statistical analysis showed that there was a highly significant correlation (r = 0.99) between the number of syncytia observed (SIU) and the number of LB59Ly cells seeded (Xi). Fig. 3 shows that the regression curve after logarithm transformation

\[
\log \frac{Y}{X} \text{ or } \log \frac{\text{SIU}}{X} = \log \frac{X}{X_d}
\]

in which X represents the maximum amount of LB59Ly cells seeded and Xd the number of LB59Ly cells seeded at dilution d, is horizontal, thus indicating a single-hit kinetics for syncytial induction. These findings suggest that as with FLK-BLV cells, only one LB59Ly cell is sufficient to induce one syncytium. Furthermore, the constancy of the ratio Y/X indicates that all LB59Ly cells are equivalent with respect to their syncytia-inducing activity. Therefore, all LB59Ly cells are infected and produce BLV antigens.

**Early polykaryocytosis inhibition test (EPI)**

In the EPI test, LB59Ly cells or FLK-BLV cells were preincubated with normal bovine serum or with that of enzootic lymphosarcoma. The ability of LB59Ly cells to induce early polykaryocytosis is specifically inhibited by the EPI-positive serum, thus indicating that the antibodies detected in the EPI tests recognized the same antigens in both cell lines.

**Infectivity of the virus released by established LB59Ly cells**

The late syncytia-inducing activity of the virus released in the culture medium of LB59Ly cells was investigated after infection of BESP, LyNX or MOL cells. A control experiment was performed with BLV released by FLK-BLV cells. It was found that the three cell lines were sensitive to the syncytia-inducing activity of both viruses.

**DISCUSSION**

The FLK-BLV cells were the first to be obtained which released large amounts of BLV. Their availability permitted the rapid development of bovine leukaemia research. However, they have an ovine origin. Such heterologous infections with retroviruses have been shown in many instances to favour the rescue of endogenous, otherwise non-expressed, retroviruses specific for the exogenously infected species. Heterologous infections were also demonstrated
to be the cause of recombinational events with endogenous viruses or with cellular genetic
sequences. In all these instances, the viral progeny is slightly different from the originally
infecting particles and this can affect the antigenicity of the viral proteins to a large extent.

In view of these considerations, great care should be taken in the interpretation of data
obtained with antigens prepared in heterologous cells, and the quality of the preparations
should be reassessed from time to time. Particular attention should be given when
fundamental studies are undertaken using minor viral antigens and virus-induced cellular
antigens. The results reported here could aid in overcoming such theoretical or practical
difficulties. The LB59Ly cell line may be of interest in virological and immunological studies
concerning bovine leukaemia as the cells were naturally infected with BLV.

Several authors have previously reported on the establishment of bovine lymphoid cell lines
with reference to bovine lymphosarcoma (Cornefert-Jensen et al., 1969; Van der Maaten et al.,
1974; Ressang et al., 1974; Onuma et al., 1976; Onuma & Olson, 1977). However, for
reasons which are still unclear, even when such lymphoid cell lines could be established, they
were found to produce either no or only small amounts of BLV. In contrast, LB59Ly cells,
even after their permanent establishment, were shown to produce high amounts of virions at a
level comparable to that of FLK-BLV cells.

The identity of the viruses released by LB59Ly cells or by FLK-BLV cells with respect to
their major properties was established on the following grounds. LB59Ly virus has a density
of 1.16 g/ml, it contains an RNA molecule and an RNA-dependent DNA polymerase
endogenous activity highly resistant to actinomycin D (reverse transcriptase); this enzyme
displays a higher activity in the presence of Mg$^{2+}$ cations than in the presence of Mn$^{2+}$ cations
and is therefore apparently identical to the enzyme of the virions produced by FLK-BLV
cells.

These results were further confirmed by PAGE analysis. All the detectable proteins of
FLK-BLV-released virions were also present in the preparations of LB59Ly virus and their
migration patterns were identical. This pattern, gp51, p24, p15, p12 and p10, was similar to
that previously described by Ghysdael et al. (1978) and in this respect bovine-borne BLV
cannot be distinguished from BLV produced by FLK-BLV cells. The antigenicity of LB59Ly
virus was also compared to that of FLK-BLV virus. Their identity was found to be complete
in immunodiffusion tests and in competitive radioimmunoassays with purified gp51 or p24. A
more detailed analysis of the antigenic properties of smaller proteins has not yet been possible
due to difficulties encountered in their isolation and purification in sufficient amounts.

LB59Ly cells also induced early polykaryocytosis in mixed cultures with the same
efficiency as FLK-BLV cells and this phenomenon could be inhibited by an EPI-positive
serum. It may thus be concluded that BLV antibodies recognize BLV-induced antigens in the
two cell lines. In these conditions the EPI test could be modified by use of LB59Ly cells.

In conclusion, the results presented above allow the use of LB59Ly cells for a number of
studies in which natural BLV infection and production may be considered as an important
aspect.

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