Restricted viral cDNA synthesis in cell lines that fail to support productive infection by bovine leukemia virus

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Abstract

Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leucosis, which results in significant economic losses on many affected farms. BLV infects a wide range of animals as well as cell lines derived from various mammalian species and organs; however, studies show that only some cell lines support sustained production of viral progeny. The differences between cells that produce viral progeny and those that do not are unclear. The aim of this study was to identify the steps of BLV replication that are associated with the capacity of a cell to support a productive infection. Eleven cell lines derived from various species were categorized into two groups, those that produce BLV progeny and those that do not, and the efficiency of viral attachment was compared. In addition, viral entry and reverse transcription were compared for two BLV-producing cell lines and three non-producing cell lines. BLV attached to and entered all of the tested cells. However, synthesis of viral DNA was inhibited in all three non-virus-producing cell lines, suggesting that BLV production was blocked either prior to or at the stage of reverse transcription. These results increase our understanding of the BLV life cycle and should enable better control over the spread of BLV.

Introduction

Bovine leukemia virus (BLV) is a delta retrovirus that is closely related to human T cell leukemia virus (HTLV). About 30% of BLV-infected cattle show persistent lymphocytosis (PL), and some develop a B cell lymphoma called enzootic bovine leukemia (EBL) 5–10 years after infection. Although EBL occurs in less than 5% of infected cattle, infected asymptomatic cattle and cattle with PL also cause economic losses for affected farms [1, 2].

The natural host of BLV is the cow; however, antibodies against BLV have been detected in domestic animals such as sheep, water buffalo, and alpaca [3, 4]. Recently, the possibility of BLV infection in humans [5], and an association between BLV and human breast cancer, has been suggested [6–10], although it is unclear how much BLV contributes to cancer in humans [11, 12]. Furthermore, various experimentally infected mammalian species and chickens show seroconversion [3, 13–17].

The host range of BLV has also been studied using cell lines. These studies showed a wide range of BLV infection [18–20]. By contrast, a study of wild-type BLV revealed sustained production of progeny virus only in bat lung cells and canine thymus cells [18]. These results suggest that infection can proceed up to a certain point in a variety of cells, but that BLV can complete the replication cycle only in some host cells. It is not clear which steps of the BLV replication cycle are inhibited in non-permissive cells. Although elements of BLV infection and replication are being studied, the receptors and many other cellular factors involved in BLV infection and replication remain unclear [21–23].

This study examined the capacity of cells derived from various mammalian species to produce progeny virus. The efficiency of early steps of BLV infection (i.e., BLV attachment, BLV entry, and viral cDNA synthesis) was compared in each cell type. We found that synthesis of viral cDNA was inhibited in cell lines that did not produce progeny virus.

Materials and Methods

Cells and viruses

MDBK cells [bovine kidney; American Type Culture Collection (ATCC) CRL6071] were purchased from RIKEN Bio Resource Center. SIRC cells (rabbit) [24] were kindly provided by Dr. A. Takase (SOKA University, Japan). BT (bovine turbinate; ATCC CRL-1390), EBTr (bovine trachea; ATCC CCL-44), NIH3T3 (mouse embryonic fibroblast; ATCC CRL-1658), SC-1 (mouse embryonic fibroblast; ATCC CRL-1404), NRK (rat kidney; ATCC CRL-6509), Tb1Lu (bat; ATCC CCL-88), CC81 (cat) [25], COS7 (monkey kidney; ATCC CRL-1651), HOS (human bone; ATCC CRL-1543), and FLK-BLV (fetal lamb kidney cells persistently infected with BLV) [26] cells were maintained at our institute. BT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Sigma Aldrich) containing 10% fetal bovine serum (FBS), and all other cells were cultured in original DMEM (Thermo Fisher Scientific) containing 5% FBS at 37°C in 5% CO_2.

To obtain infectious BLV, FLK-BLV cells were cultured to 50–70% confluency in 10 cm dishes. The medium was then exchanged for 8 ml of fresh culture medium, and the culture fluid was collected after 16–24 h. The culture supernatant was then passed through a 0.22 μm membrane to remove cell debris and used as a source of infectious BLV. BLV titers were measured using the syncytium formation method. To obtain high titer virus, fresh supernatants of FLK-BLV without freezing or thawing (1.3-1.6 $\times 10^4$ syncytium formation units/ml) were used for the detection of BLV produced by cells (see below). For other experiments, BLV stocks with 1.4-1.6$\times 10^3$ syncytium formation units/ml were used.

Detection of BLV produced by cells

On the day before infection, 1×10^5 cells were seeded into 6 well plates. The next day, medium was replaced with culture medium containing polybrene (final concentration, 10 μg/ml) in order to increase the infection efficiency. After 1 h, the medium was replaced with FLK-BLV cell culture supernatant or normal culture medium containing 10 μg/ml polybrene (Millipore). After 1 h, the medium was removed and the cells were cultured for an additional 7 days in fresh culture medium containing 10% FBS. Cells were passaged at about a 1:4 ratio when confluent. Seven days after virus inoculation, the culture fluid was collected, passed through a 0.22 μm membrane, and used for the NIH3T3 cell-binding assay or for the syncytium formation assay using CC81 cells (see below) [25].

For the syncytium formation assay, CC81 cells were seeded in 3.5 cm dishes at a density of 5×10^4 cells/dish. The next day, the cells were pre-incubated for 1 h with medium containing 20 μg/ml polybrene, which was subsequently replaced with culture fluid from various BLV-infected cells containing 20 μg/ml polybrene. After 1 h, the medium was removed and replaced with fresh culture medium, and the cells were

incubated for 4 days. Cells were then fixed with methanol and stained with May-Grunwald-Giemsa reagent (1:20 dilution in deionized water) (Wako). The dishes were divided into squares and the number of multinucleated cells per square was counted.

Detection of BLV attached to the cell surface

Cells were detached from culture dishes using Hank's buffer containing 0.05% trypsin and 0.2 g/L EDTA-4Na (Thermo Fisher Scientific), followed by washing first with DMEM containing 10% FBS and then with ice-cold DMEM containing 1% FBS and 0.05% NaN_3 (FACS buffer). All subsequent washes were performed with ice-cold FACS buffer, and for all centrifugation steps, cells were spun for 2 min at 600 × g at 4°C. Cells (6×10^5) were suspended in 1 ml of ice-cold culture supernatant from FLK-BLV cells and incubated for 1 h at 4°C with mild shaking. After two washes, the cells were incubated for 30 min on ice with a mouse anti-BLV envelope glycoprotein gp51 monoclonal antibody (BLV2; 1:100 dilution in FACS buffer) (VMRD). The cells were then washed twice and incubated for 30 min on ice with a biotinylated anti-mouse IgG antibody (1:100 dilution in FACS buffer) (Jackson Laboratories), followed by two washes and a final incubation for 30 min on ice with PE-conjugated streptavidin (1:100 dilution in FACS buffer) (BD Biosciences). After two final washes, the cells were fixed with phosphate-buffered saline (PBS) containing 1% paraformaldehyde, and the fluorescence intensity was measured on a flow cytometer (EPICS XL, Beckman Coulter or FACS Aria, BD Biosciences).

Detection of BLV p24 protein in cells

One day before infection, each cell line (1×10^5 cells) was seeded into 35 mm dishes with 14 mm^2 collagen-coated glass bottoms (Matsunami Garasu Kougyou). On the following day, cells were pre-incubated for 1 h at 37°C in 5% CO_2 with medium containing 10 µg/ml polybrene and then incubated with 1 ml FLK-BLV culture supernatant containing 10 µg/ml polybrene for an additional 1 h. For fixing and permeabilizing cells, IntraPrep Permeabilization Reagent (Beckman Coulter) was used. After removing the virus and rinsing the cells with FACS buffer, cells were fixed with 100 µl of Reagent 1 from the kit for 15 min at room temperature (RT). Then, the dishes were rinsed with FACS buffer again and blocked for 20 min at RT with a 4% aqueous solution of Block Ace (DS Pharma Biomedical) diluted 1:4 with FACS buffer. After blocking, dishes were rinsed with FACS buffer, 50 µl of Reagent 2 from the kit was added, and cells were incubated for 5 min at RT to permeabilize the cell membranes. Then, 50 µl of mouse anti-BLV p24 antibody (BLV3; 1:50 dilution in Reagent 2) (VMRD) was added (1:100 dilution, final), and dishes were incubated for an additional 15 min at RT. After rinsing with FACS buffer, cells were incubated for 15 min at RT with 100 µl of FITC-labeled anti-mouse IgG antibody (1:50 dilution in FACS buffer) (Immunotech)

and then washed with FACS buffer. Fluorescence was observed after mounting with 200 µl of VeltaShield (Vector Laboratories) on an Olympus IX71 fluorescence microscope.

Quantification of reverse-transcribed BLV cDNA

One day before infection, each cell line (1×10^6 cells) was seeded onto 6 cm culture dishes. The next day, cells were pre-incubated for 1 h at 37°C in 5% CO_2 in medium containing 10 µg/ml polybrene. After the cells were cooled on ice for 10 min, they were incubated with 1ml of FLK-BLV culture supernatant containing 10 µg/ml polybrene for 1 h on ice. After removing the virus, cells were immediately harvested, washed, counted, and stored as a pellet (0 h) or cultured in fresh culture medium containing 10% FBS for 4–24 h at 37°C in 5% CO_2. They were then detached from the culture dishes and washed twice with PBS. After counting the cell number, the cell pellet was stored at -80°C. Extrachromosomal DNA was extracted from cells using a previously described method [27]. Briefly, the cell pellet was thawed, suspended in Tris-EDTA buffer containing RNaseA, and lysed with SDS. Chromosomal DNA was precipitated with cesium chloride, potassium acetate, and acetic acid. Extrachromosomal DNA in the supernatant fraction was bound to QIAprep Spin columns (Qiagen) and eluted with 20 µl of H_20 after washing.

The copy numbers of BLV cDNA were measured on the QuantStudio 3 real-time PCR system (Applied Biosystems) using SYBR premix Ex Taq II (Tli RNaseH plus) (TaKaRa Bio). Reactions were performed as recommended by the manufacturer. Because the DNA concentrations, as measured by NanoDrop, were below the accurate range of detection (3–12 ng/µl), 2 µl of DNA was used for each reaction without adjusting the concentration. For DNA standards, serial dilutions of pBLV913 [28] DNA were used. The PCR conditions were as follows: 40 cycles of denaturation (95°C for 15 s), followed by annealing and extension (64°C for 30 s). Data were acquired after heating the reactions to 84°C after the extension cycle. Because the melting temperature of the target product is higher than 85°C, data acquisition at 85°C reduces the non-specific signal. The following primers specific for the R/U5 region of BLV were used for amplification: Forward (nt 327-346), 5'-agggtggttctcggctgaga-3' and Reverse (nt 511-530), 5'-tgtttgccggtctctcctgg-3'. The results are shown as the number of copies of BLV cDNA per 10^4 cells.

Results

Production of viral progeny by different BLV-infected cell lines

To detect progeny virus, mouse NIH3T3 cells, which have a high capacity to bind BLV (see below), were used. NIH3T3 cells also bound to recombinant BLV gp51 protein tagged with 6×Histidine, which could be detected by flow cytometry using a monoclonal antibody against the histidine tag (Online Resource 1). BLV bound to the surface of NIH3T3 cells was detected by flow cytometry using a monoclonal antibody specific for BLV gp51, which is a surface subunit of the envelope glycoprotein. This was then detected using biotin-labeled anti-mouse IgG and PE-conjugated streptavidin. A linear correlation between the mean fluorescence intensity (MFI) of the cells and the amount of BLV was observed in a preliminary experiment conducted using serially diluted BLV (Online Resource 2).

Eleven different mammalian cell lines that were infected with BLV and then cultured for 7 days were examined for viral production. Progeny virus in the culture supernatant was detected by flow cytometry after binding to NIH3T3 cells. The 11 cell lines could be divided into two groups: those that supported production of progeny virus (detectable by the NIH3T3 cell-binding assay) and those that did not. The former included bovine BT cells and MDBK cells, bat Tb1Lu cells, cat CC81 cells, and human HOS cells. The latter included bovine EBTr cells, mouse NIH3T3 cells and SC-1 cells, rat NRK cells, rabbit SIRC cells, and monkey COS7 cells (Fig. 1a). Herein, we refer the former group of cells as “progeny-producing cells” and the latter as “non-producing cells”.

To confirm the cells’ capacity to support the production of infectious progeny virus, we chose three progeny-producing cell lines (BT, MDBK, and CC81) and three non-producing cell lines (NIH3T3, COS7, and SIRC), and tested them in a syncytium formation assay using cat CC81 cells, which generate multinucleated cells when infected with BLV [25]. Typical multinucleated cells were observed when cat CC81 cells were inoculated with cell-free culture fluid from BLV-infected BT cells or CC81 cells (Fig. 1b). Average numbers of syncytia per square were 5.5 and 4.0 for culture fluids from BLV-infected BT cells and CC81 cells (equal to 1.9×10^2 and 1.5×10^2 per dish), respectively. Culture fluid from FLK-BLV induced 34 syncytia per square when used at a 1:5 dilution. However, culture fluid from BLV-infected bovine MDBK cells did not induce syncytia in CC81 cells, despite their ability to produce progeny BLV in the NIH3T3-binding assay. NIH3T3, COS7, and SIRC cells did not make syncytia with CC81 cells.

Viral attachment to the surface of different cell lines

We next compared the efficiency of different BLV replication steps between progeny-producing and non-producing cell lines. The efficiency of BLV binding to the surface of 11 cell lines was analyzed by flow cytometry with a mouse anti-BLV gp51 antibody. FLK-BLV cells were used as a control. The MFI

of cells incubated with (shaded) or without (non-shaded) BLV are shown in Figure 2. The capacity for virus production shown in Figure 1 is also listed to the left of the histograms in Figure 2. All 11 cell lines bound BLV but FLK-BLV cells did not bind BLV, probably due to receptor interference. NIH3T3 and SC-1 cells, which are not derived from cattle, bound particularly high levels of virus, but did not produce progeny. Among the bovine cell lines, only BT cells showed an intermediate level of BLV binding; MDBK and EBTr cells showed a very low level of binding. Overall, the ability of progeny-producing and non-producing cells to bind virus did not significantly differ.

Viral entry into different cell lines

In subsequent experiments, the two cell lines that were confirmed to produce infectious progeny BLV (CC81 and BT) and three non-producing cell lines (NIH3T3, COS7, and SIRC) were examined further. For the non-producing cells, cells that showed different levels of BLV binding were selected.

To examine viral entry, the five cell lines were infected with BLV for 1 h, and intracellular viral p24 gag protein was visualized after fixation and permeabilization by staining with a mouse monoclonal antibody against BLV p24 and a FITC-labeled anti-mouse IgG (Fig. 3). Intracellular BLV p24 was observed in all five cell lines, mainly in the cytosol, and there was no difference in the localization pattern among the cell lines.

Viral cDNA synthesis in different cell lines

We next examined the efficiency of viral cDNA synthesis in the five cell lines. To measure reverse-transcribed cDNA prior to integration, extrachromosomal DNA was extracted by the modified Hirt's procedure [27] from cells that were incubated with BLV on ice to bind virus on their surface, and then incubated at 37°C for 4–24 h so that viral infection could occur. The number of BLV cDNA copies was measured by real-time PCR with a primer set for an early reverse transcript (Fig. 4). In the case of CC81 and BT cells, both of which were progeny-producing, the amount of viral cDNA exceeded 600 copies/10^4 cells at 4 h, and the levels remained at over 400 copies/10^4 cells at 24 h. However, non-producing NIH3T3 and COS7 cells harbored less than 200 copies/10^4 cells at the peak time point (4 h), and less than 50 copies/10^4 cells at 24 h. SIRC cells had less than 35 copies/10^4 cells at all time-points tested.

Discussion

Here, we examined the early stages (i.e., attachment, entry, and reverse transcription) of the BLV replication cycle and found that the only difference between progeny-producing and non-producing cells was the efficiency of reverse transcription.

Cell lines were divided into progeny-producing and non-producing groups; however, MDBK cells showed dual characteristics in that they appeared to produce progeny virus by the NIH3T3-binding assay, but it did not form syncytia on CC81 cells. To ascertain whether this unexpected character was specific to MDBK cells, we conducted further experiments on syncytium formation by Tb1Lu and HOS cells, which showed progeny-producing ability in the NIH3T3-binding assay, and confirmed that culture fluid from BLV-infected these cells could induce syncytium formation with CC81 cells (data not shown). In our procedure, the syncytium formation assay was more than 100-fold less sensitive than the NIH3T3 cell-binding assay; thus, it is not clear whether the amount of progeny virus produced by MDBK cells was simply below the detection limit of our syncytium formation assay or whether MDBK cells do not produce infectious progeny. Further study is needed to characterize MDBK cells.

BLV attached to all tested cell lines, although the amount of attached virus varied (Fig. 2). The bovine cells did not show a high level of BLV binding despite being a natural host; especially MDBK and EBTr cells showed very low binding. In a previous study, genetic variation of BLV was observed after its replication in sheep-derived FLK cells [29]. It is possible that the BLV used in this study had become adapted to sheep, and that a fresh field isolate of BLV from cow would have bound more bovine cells. Although no cellular receptor for BLV has been identified to date [30–32], our findings are consistent with those of a previous study, showing that an N-terminal region in the envelope glycoprotein that is homologous to the receptor-binding domain of the HTLV envelope binds to cell lines derived from various mammalian species [33].

Furthermore, BLV entered all five of the cell lines tested, and there was no observable difference in the localization of BLV p24 protein among the cells (Fig. 3), suggesting that BLV can penetrate into the cytoplasm of all these cell lines. To confirm the intracellular localization of p24, we tested whether surface protein was stained after fixation and permeabilization treatment. BLV gp51 protein on the BLV-infected cells was detected clearly using a monoclonal antibody against gp51 without fixation and permeabilization treatment, but was not detected after the treatment as noted in the manufacturer's instructions for the reagent (data not shown), suggesting that p24 protein stained with the antibodies are not one on the surface.

Extrachromosomal DNA has been used to identify viral DNAs in infected cells because the fraction of viral DNA is much larger in extrachromosomal DNA than in total DNA [34]. In this study, to monitor

the viral reverse transcripts prior to integration, the modified Hirt's procedure [27] was used to extract extrachromosomal DNA. The modified procedure can extract small DNAs in the linear form as well as in the circular form [27]. Primers were positioned in the 5'-long terminal repeat, which allows the detection of the early products of reverse transcription [35]. The amount of early viral cDNA transcripts in the extrachromosomal DNA clearly differed between BLV-producing and non-producing cell lines. To rule out viral cDNA contamination from the BLV-containing culture supernatant, cells incubated with BLV on ice but not cultured at 37°C were used as the first time-point (0 h). Relatively little viral cDNA was detected at 0 h in all of the cell lines tested (less than 5.5 copies/10^4 cells). Previous studies have shown that cells from various species can be infected with BLV and produce virus-derived protein; however, the reported efficiencies are not consistent [18–20]. Here, we observed low levels of reverse transcription in non-producing cells, which is consistent with these results.

In many cases, viral cellular tropism is determined by the expression of virus receptors. This work shows that BLV can bind a wide range of cells and that the ability of cells to produce BLV progeny is determined by factors operating either prior to or at the start of reverse transcription. Studies of primate lentiviruses and murine retroviruses suggest that several host restriction factors affect virus replication (reviewed in [36–39]). It is possible that such cellular factors are associated with replication of BLV as well. The findings reported here are not detailed enough to help identify the factors, but rather justify further study for this.

Acknowledgements

We thank Dr. D. Derse (National Cancer Institute at Frederick, USA) for providing the pBLV913 plasmid and Dr. A. Takase (SOKA University, Japan) for providing the SIRC cells. We also thank Dr. K. Nishigaki and Dr. A. Miyake (Yamaguchi University, Japan) for valuable discussions.

Funding

This research did not receive any grants from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Captions

Fig. 1 Amount of progeny virus produced by BLV-infected cells

a BLV production measured in an NIH3T3 cell-binding assay. Progeny virus produced by BLV-infected cell lines derived from various mammalian species (shown on the left) was detected by flow cytometry after absorption to NIH3T3 cells, followed by staining with a mouse anti-gp51 antibody, a biotin-labeled anti-mouse IgG antibody, and PE-labeled streptavidin. The mean fluorescence intensity of 10,000 NIH3T3 cells was analyzed in each sample. FLK-BLV is a BLV-producing cell line used as a positive control. The control sample shows the background staining of antibodies and PE-labeled streptavidin in the absence of BLV. Three independent experiments for BT, MDBK, NIH3T3, SIRC, CC81, and COS7 cells, and two independent experiments for the other cell lines were done using EPICS XL or FACS Aria. All experiments yielded similar results. A representative result obtained using EPICS XL is shown.

b Syncytia formation in CC81 cells by BLV produced from BT, CC81, and FLK-BLV cells. CC81 cells were cultured for 4 days with culture supernatant from BLV-infected cells. Multinucleated cells were observed after fixation and Giemsa staining.

Fig. 2 Relative amounts of BLV attached to the cell surface

Cell lines derived from various mammalian species (shown on the left) were incubated with culture supernatant from FLK-BLV cells, which contains infectious BLV virions. BLV attached to the cell surface was detected by flow cytometry using FACS Aria after staining with a mouse anti-gp51 antibody, a biotin-labeled anti-mouse IgG antibody, and PE-labeled streptavidin. The mean fluorescence intensity of 10,000 cells was analyzed and the averages of three independent experiments are shown in the bar graphs. Unshaded graphs show the background binding of antibodies and PE-labeled streptavidin in the absence of BLV. The bars show the standard errors of three independent experiments. For FLK-BLV cells, the result of one experiment is shown. The capacity of each cell line to maintain virus production is also shown.

Fig. 3 Localization of BLV p24 capsid protein

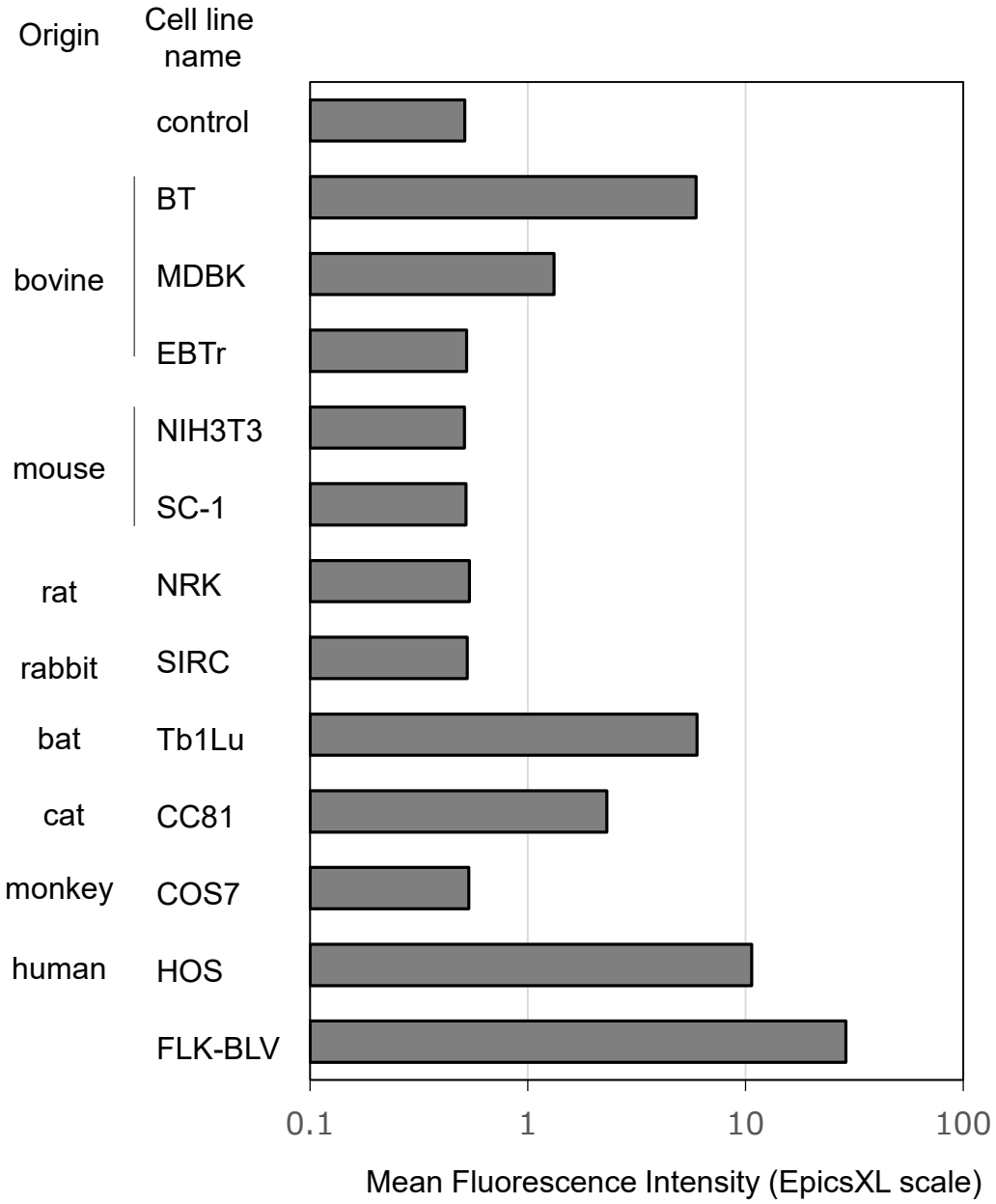
Cells were infected BLV for 1 h, and intracellular BLV p24 capsid protein was labeled with a mouse anti-BLV p24 antibody and FITC-labeled anti-mouse IgG after fixation and permeabilization of the cells. The FITC signal was observed by fluorescence microscopy (Olympus IX71) at 320× magnification. Uninfected: background staining of antibodies in the absence of BLV.

Fig. 4 Quantification of BLV cDNA

Cells were incubated with culture supernatant from FLK-BLV cells, which contains infectious BLV, for 1 h on ice. After 0, 4, 12, and 24 h of incubation at 37°C, cells were counted and extrachromosomal DNA was extracted using the modified Hirt's method [27]. The BLV cDNA copy number was measured by real-time PCR. The results obtained from BLV-producing cell lines are shown by solid symbols: CC81 (solid squares) and BT (solid diamonds) and those from non-producing cell lines are shown by open symbols: NIH3T3 (open squares), COS7 (open diamonds) and SIRC (open circles). The bars show the standard error of experimental triplicates.

Fig. 1

a


Origin
Cell line name
control
bovine
BT
MDBK
EBTr
mouse
NIH3T3
SC-1
rat
NRK
rabbit
SIRC
bat
Tb1Lu
cat
CC81
monkey
COS7
human
HOS
FLK-BLV
0.1
1
10
100
Mean Fluorescence Intensity (EpicsXL scale)

b

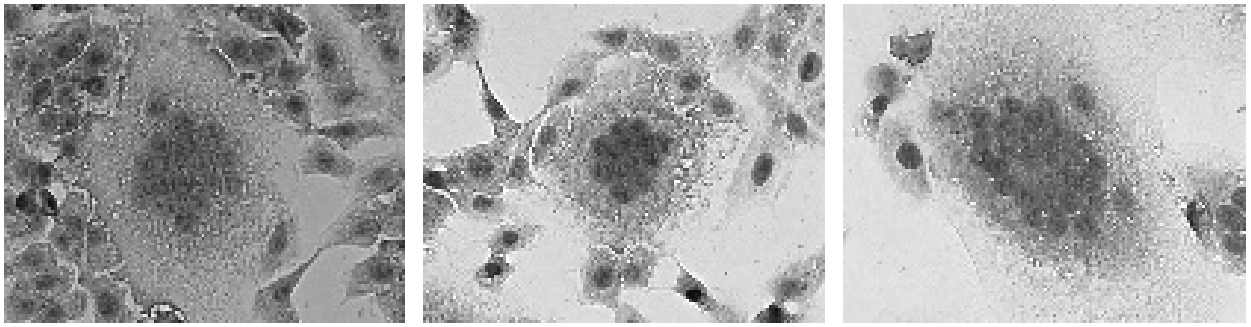

Culture supernatant:
BT
CC81
FLK-BLV

Fig. 2

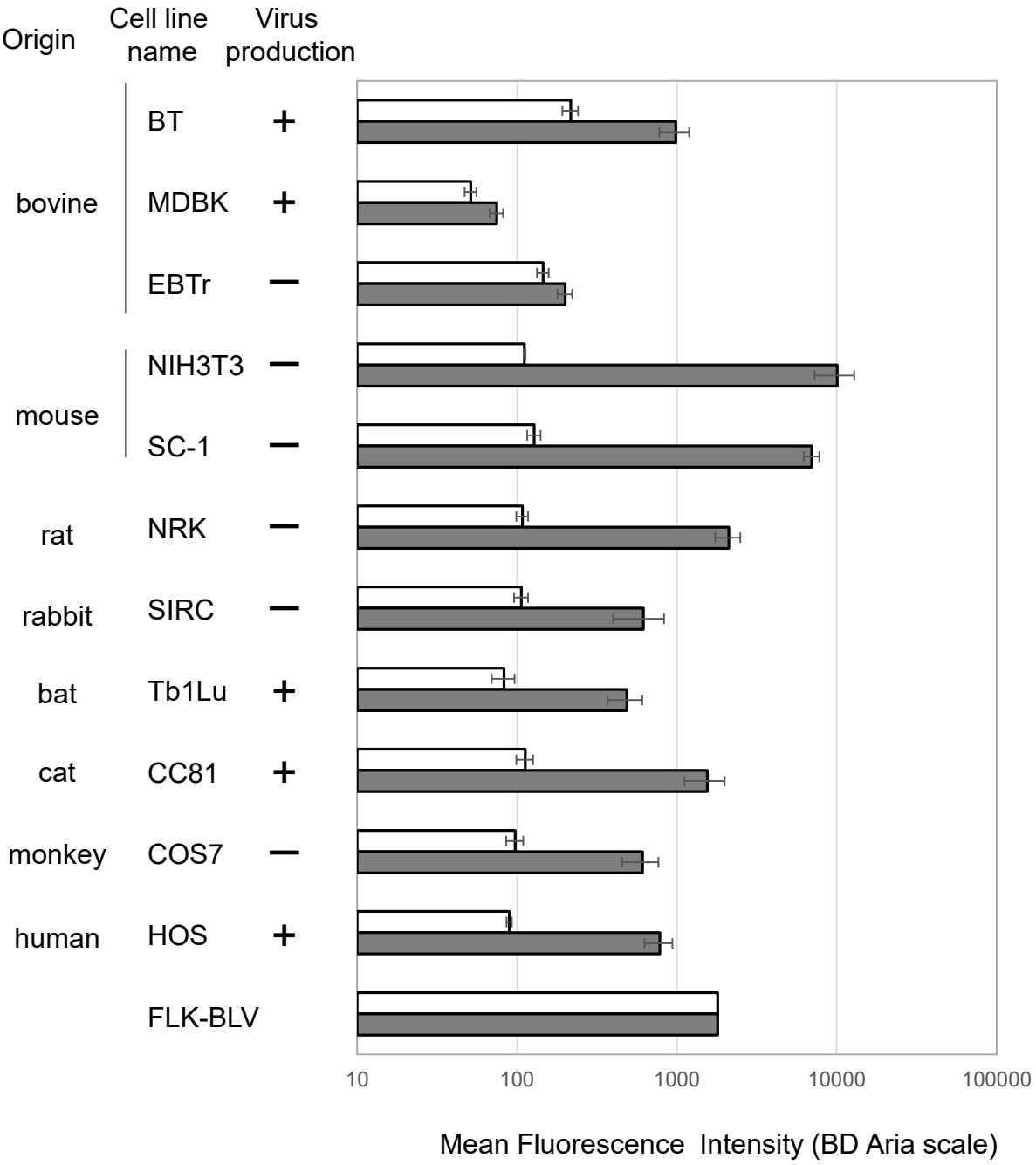

Origin
Cell line name
Virus production
bovine
BT
+
MDBK
+
EBTr
−
mouse
NIH3T3
−
SC-1
−
rat
NRK
−
rabbit
SIRC
−
bat
Tb1Lu
+
cat
CC81
+
monkey
COS7
−
human
HOS
+
FLK-BLV
10
100
1000
10000
100000
Mean Fluorescence Intensity (BD Aria scale)

Fig. 3

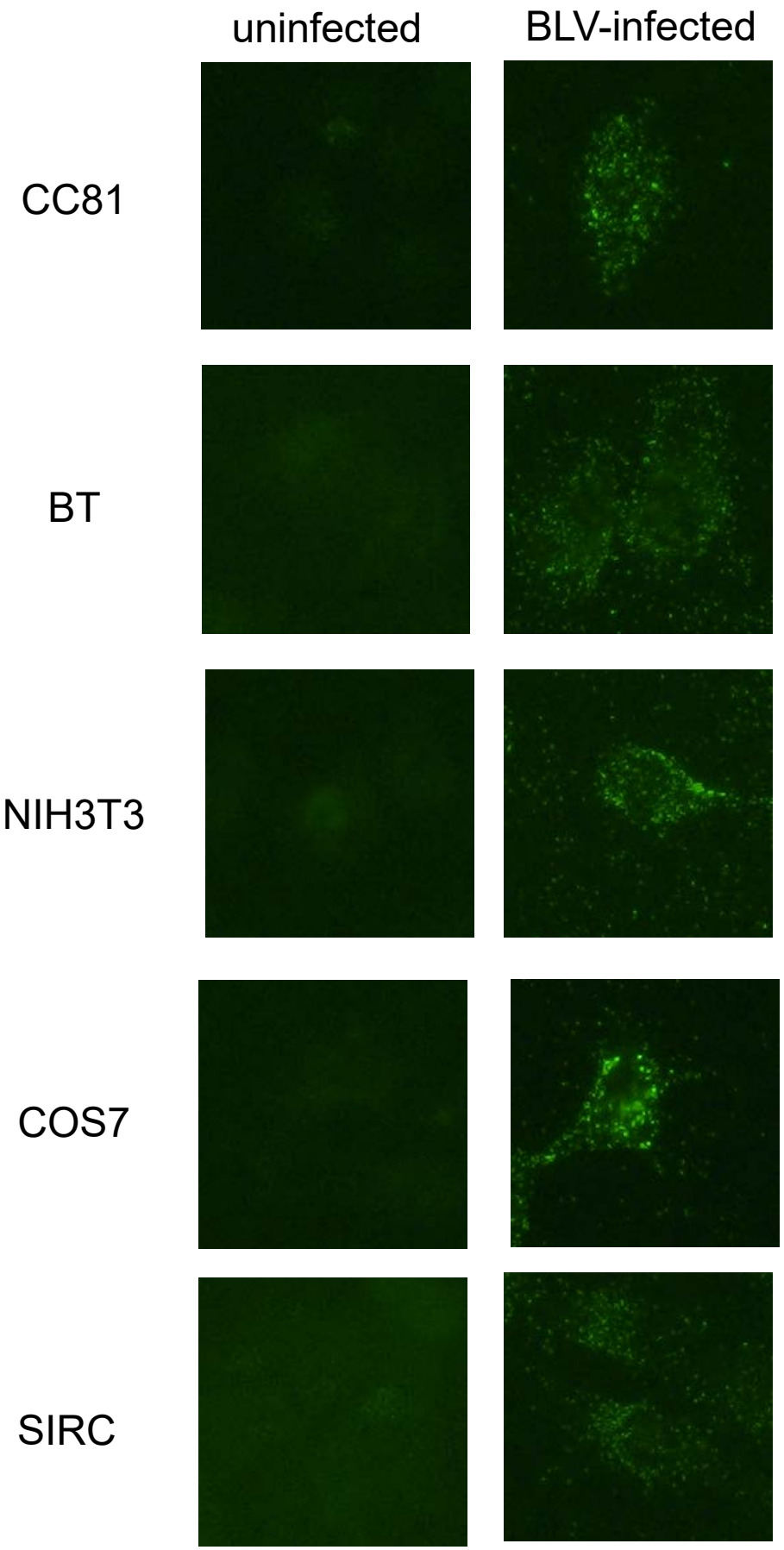
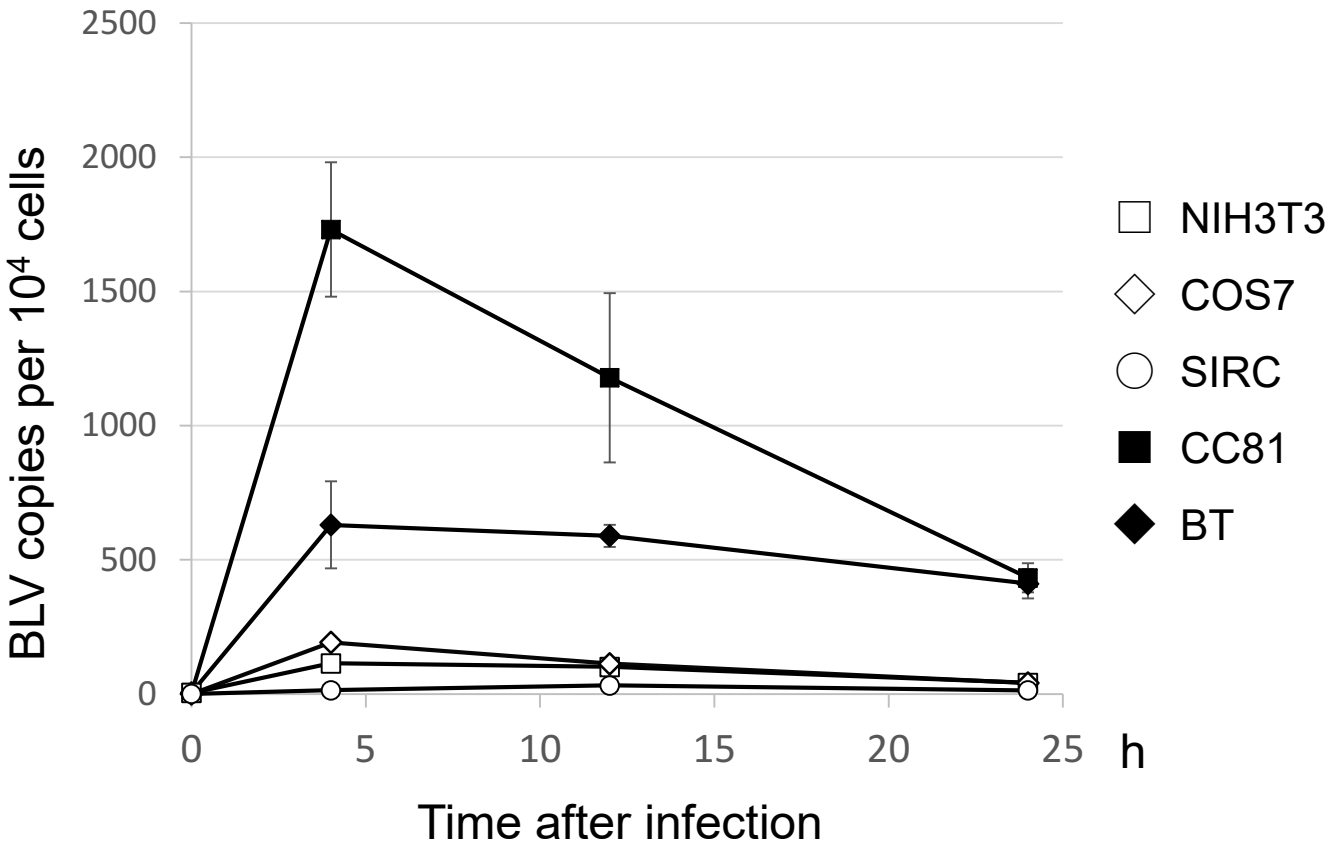


Fig. 4


2500
2000
1500
1000
500
0
BLV copies per 10^4 cells
0
5
10
15
20
25
h
Time after infection
NIH3T3
COS7
SIRC
CC81
BT