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Research Article

Bovine Leukemia Virus Non-Productive Infection of Human Mammary Epithelial Cells (MCF10A)

Lucía Martínez Cuesta[1], Pamela A Lendez[3], Maureen A Sheahan[2], Raymond RR Rowland[2], Maria Victoria Nieto Farias[3], Guillermina L Dolcini[3] and Maria Carolina Ceriani[3*]

[1]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET- CIVETAN), Buenos Aires, Argentina

[2]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas, USA

[3]Laboratorio de Virología, Centro de Investigación Veterinaria de Tandil (CIVETAN, CONICET-CICPBA), Facultad de Cs. Veterinarias, UNCPBA, Buenos Aires, Argentina

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ABSTRACT

Bovine leukemia virus (BLV) is a retrovirus that causes lymphosarcoma in cattle. Some researchers suggest that BLV could be related to breast cancer development, however, evidence that the virus can infect the human counterpart is lacking. For that reason, the objective of this study was to infect *in vitro* a human mammary epithelial cell line (MCF10A) with BLV. The results suggest that the infection is non-productive, since only a fragment of the viral gene *pol* was detected in the cellular DNA. These results are consistent with previous studies, where fragments of different BLV genes were found in human mammary tissue. Future studies should investigate whether this non-productive infection can be associated with human breast cancer.

Impacts

Bovine leukemia virus can infect a human mammary epithelial cell line and is integrated as a provirus. BLV infection on human mammary epithelial cell line (MCF10A) is not productive. BLV non-productive infection of the human mammary epithelial cells can be involved in breast cancer development.

Introduction

Bovine leukemia virus (BLV) is a δ-retrovirus that affects cattle causing a lymphoproliferative disease. Its main target cell is the B lymphocyte but, naturally and experimentally, this virus can infect other cells such as neutrophils, epithelial and brain cells [1-4]. Since the detection of BLV in bovine epithelial mammary cells, researchers have considered the possibility of this virus infecting human epithelial mammary cells [2, 5, 6]. Many facts support this idea. First, BLV belongs to the same viral family that the human T lymphotropic virus (HTLV). This is a carcinogenic virus in humans and shares structural and functional homology with BLV [7]. Although the BLV receptor in cattle is not yet elucidated, it is believed that it is a protein from the adaptor-related protein complex 3 (AP-3) [8]. This protein is also present in humans, who have four different AP complex. The fact that the putative receptor of the virus has 88% homology with the human counterpart and that the putative binding site for the viral protein gp51 is completely homologous in both species support the possibility of BLV infecting humans [9].

Virus have been associated with the development of different tumors in humans. It is believed that around 12% of the cancer cases are from viral origin [10]. The possible role of BLV in the development of human breast cancer has been investigated in the past years with controversial results [11-15]. Numerous studies have detected fragments of viral genes and proteins in human breast samples using different techniques such as

Correspondence to: Maria Carolina Ceriani, Laboratorio de Virología, Centro de Investigación Veterinaria de Tandil (CIVETAN, CONICET-CICPBA), Facultad de Cs. Veterinarias, UNCPBA, Pinto 399, Tandil 7000 Pcia. Buenos Aires, Argentina; E-mail: cceriani@vet.unicen.edu.ar

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in situ PCR, immunohistochemistry, and conventional PCR [13-18]. Most of these studies suggest that the virus might be implicated in the onset or development of human breast cancer. However, to our knowledge, no study unequivocally demonstrates whether the virus can infect human breast cells. Confirming that the virus can infect human mammary cells is the first step to analysing the possible relationship between the virus and the development of human breast cancer. Thus, the aim of this study was to infect *in vitro* a human mammary epithelial cell line (MCF10A) and analyse its effects.

Materials and Methods

BLV was obtained from BLV-infected adult cows in the Manhattan area (Kansas, USA). From each animal, 20 ml of heparinized blood (5 U ml^{-1}) were obtained by jugular venipuncture. Peripheral blood mononuclear cells (PBMCs) were isolated using SepMate™ (STEMCELL Technologies Inc, Canada) and Ficoll-Paque™ PREMIUM (GE Healthcare) following the manufacturer´s instructions. PBMCs were cultivated in RPMI supplemented with 10 % FBS by incubating at 37°C with 5% CO_2 until use.

For MCF10A infection, forty thousand cells were seeded in each well of a six well plate and cultured until they reached 90% confluence. The cells were co-cultured with 1 x 10^5 PBMCs from the infected animals for 24 h. After that time, cells were passaged three times (to passage 3 post infection; 3 ppi) and started to be analysed. The experiment was conducted in triplicate using PBMCs from three different animals. The infected cell line was designated MCF10A BLV. DNA was extracted from cell pellets using Qiagen columns (QIAamp DNA Mini Kit, Germantown, MD, USA) according to the manufacturer's protocol. DNA concentration and purity were determined by absorbance at 260 nm and 260/280 coefficient, respectively, in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Purified DNAs were stored at -20 ºC until use.

Obtained DNA was used as a template to amplify the viral gene *pol*, as well as the human and bovine GAPDH by conventional PCR. All three PCR reactions were performed in a final volume of 25 µl containing 12.5 µl of GoTaq MasterMix (Promega®), 0.4 mM primers and 1 µl of the template. The primers and PCR program used for *pol* and hGAPDH were described previously [16, 19]. For bGAPDH amplification the primers used were F: 5´ TGTTGTGGATCTGACCTGCC 3´ R:5´ TGAAGGGCTGTTTACCGAGC 3´ and the amplification was performed 2 min at 95°C, 35 cycles of 30 s 95°C, 30 s 50°C and 22 s 72°C ending with 5 min 72°C. The PCR products and a 1 Kb DNA ladder were run in a 1% agarose gel and visualized using ethidium bromide.

RNA was extracted from cell culture supernatants and pellets using MagMAX™-96 Viral RNA Isolation kit (Applied Biosystems, USA) following the manufacturer's instructions. Obtained RNA was used as a template to amplify the viral gene *pol* by RT PCR using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, Thermo Scientific, Waltham, MA, USA) and the primers described previously [19]. The program included a cycle of 30 min 50°C and 2 min 94°C; followed by 8 cycles of 1 min 94°C, 1 min 55°C and 1 min 72°C; 25 cycles of 1 min 94°C, 1 min 62°C and 1 min 72°C and a final extension of 10 min72°C. The PCR products and a 1 Kb DNA ladder were run in a 1% agarose gel and visualized using ethidium bromide. For DNase treatment, DNase I, RNase-free (Thermo Scientific, Waltham, MA, USA) was used, following the manufacturer´s instructions.

In order to detect the viral protein p24 in cell culture pellets and supernatants, a western blot was performed. Cell cultures pellets were lysated using 20 mM Tris-HCl, pH 7.5 and heated at 100°C for 10 min. Cell culture supernatants and lysates were run in 12% w/v denaturalizing polyacrylamide gels and transferred onto Amersham™ Hybond™ 0.2 µm PVDF (GE Healthcare Life Sciences). After 1 h blocking with MTBS, the membrane was incubated 1 h with an anti-BLV p24 monoclonal antibody (1:100) (NIH AIDS Reagent Program), washed 3 times for 15 min each with PBS-Tween and incubated with 1:500 peroxidase labeled goat anti-mouse IgG (Seracase) for 1 h. The membrane was washed with PBS and incubated for 5 min with CN/DAB substrate 10x (Thermo Fisher Scientific) to develop the reaction and stopped by rinsing with tap water.

Cell viability was analysed using MTT. For that purpose, ten thousand cells were seeded in each well of a 96 well plate and incubated 48 h at 37°C. Twenty microliters of MTT solution (5 mg ml^{-1}) (Sigma-Aldrich) were added to each well. After 4 h incubation, the plate was centrifuged at 2000 rpm 2 min and the formazan crystals were resuspended using 200 µl of DMSO (MP Biomedicals). The absorbance was determined at 570 nm using an Epoch Microplate Spectrophotometer (BioTek). Results are express as % of the non-infected MCF10A cells. The experiment was repeated 3 times with four technical replicates each time. The statistical analysis was performed in R using ANOVA test.

Results and Discussion

Our results show that human mammary cell line MCF10A can be infected with BLV *in vitro*. The new cell line called MCF10A BLV resulted from the infection of MCF10A with PBMC from highly BLV infected cows. The analysis of this new cell line began at passage 3 ppi when genomic DNA was extracted, and a fragment of the viral gene *pol* was detected by conventional PCR. DNA obtained from MCF10A BLV and the positive control amplified a unique fragment of the expected size of 184 pb (Figure 1). Moreover, when using MCF10A BLV DNA as a template, only human and not bovine GAPDH could be amplified, dismissing the possibility that the fragment of the *pol* gene detected in MCF10A BLV cells might come from remaining bovine PBMCs used for infection.

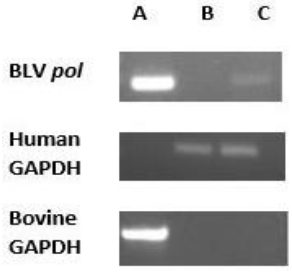

Figure 1: A fragment of BLV *pol* gene and human GAPDH was amplified in MCF10A BLV genomic DNA. **A)** PBMCs DNA from a naturally infected cow used for MCF10A infection, **B)** MCF10A DNA, **C)** MCF10A BLV DNA.

The 24 kDa viral protein was not detected in cells supernatant which suggest that the new cell line does not release viral particles into the cell culture supernatant. Considering the possibility that the viral protein p24 was synthesized but unable to be released, cell pellets were lysed for analysis. The protein was not detected by Western blot in the cell lysates nor was it found in MCF10A-BLV supernatant (Figure 2a). The absence of this viral protein in cell culture supernatants was confirmed up to 30 ppi.

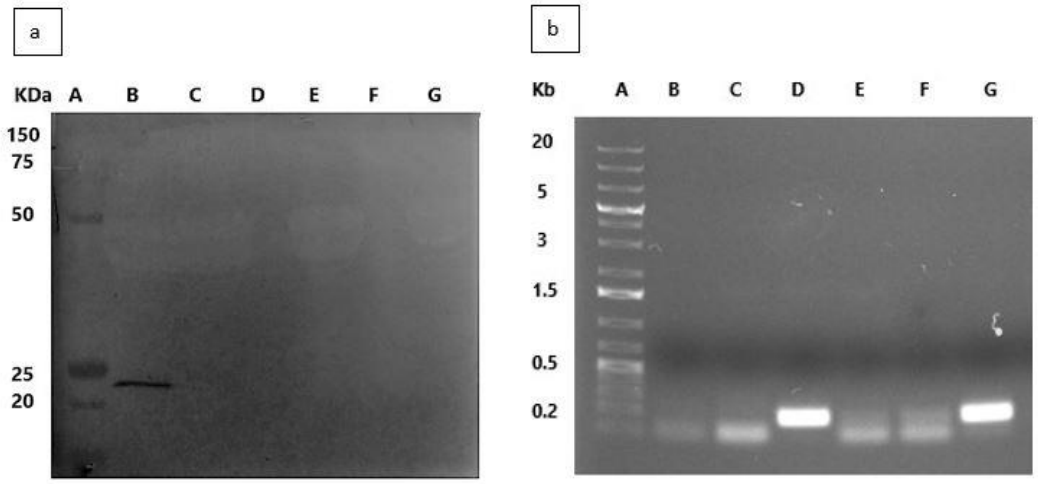

Figure 2: a) Western blot analysis of viral protein p24 in cell culture supernatants and pellets lysates. **A)** MW marker. **B)** positive control, MAC-T BLV 32ppi supernatant. **C)** MCF10A BLV 24ppi supernatant **D)** MCF10A BLV 24ppi pellet lysate. **E)** MCF10A BLV 18ppi supernatant. **F)** MCF10A BLV 18 ppi pellet lysate. **G)** MCF10A pellet lysate. **b)** Viral RNA is not detected in MCF10A BLV cell pellets. RT-PCR was performed to detect viral gene *pol* in cell pellets. **A)** Molecular weight marker. **B)** pellet MCF10A BLV 26 ppi treated with DNase. **C)** pellet MCF10A BLV 16 ppi treated with DNase. **D)** pellet MAC-T BLV 19 ppi treated with DNase. **E)** pellet MCF10A BLV 26 ppi. **F)** pellet MCF10A BLV 16 ppi. **G)** pellet MAC-T BLV 19 ppi.

To confirm that the cells were not releasing virus, an RT-PCR assay was carried out to detect RNA from the viral gene *pol* in cell culture supernatants and pellets. No amplification was observed in the supernatants (data not shown). To analyse the presence of the viral gene in cell pellets, a kit that extracted both viral DNA and RNA was used (MagMAX™-96 Viral RNA Isolation kit). When these nucleic acids were analysed by RT-PCR, the fragment of a viral gene *pol* was amplified. However, when a DNase treatment was carried out before the RT-PCR, amplification of the *pol* fragment was no longer detectable (Figure 2b). Cell viability analysed by MTT showed no difference between BLV infected and uninfected MCF10A ($p = 0.2857$) for at least 33 ppi. It is known that non-productive infections could play a role in tumor development [20]. Although the virus cannot generate new infective viral particles, it can produce proteins that interfere with the normal cell cycle.

Our results show that BLV infected human epithelial breast cells have no difference in cell viability compared to the uninfected cells when measured by MTT. The reason for this lack of effect on the cell viability could be that the cells were not infected for enough time to suffer significant changes. Previous studies that analysed the presence of the virus in human mammary tissue found that the viral DNA fragments were present in healthy individuals up to 10 years before the development of breast cancer [14]. This suggests that if BLV does play a role in breast cancer tumorigenesis, it likely progresses very slowly and the effect of the virus on cell viability will not be evident for a long time. Moreover, in most virus-related cancer, the presence of the virus is not the only condition necessary to develop the tumor. Other factors such as immunosuppression, chronic inflammation, and spontaneous mutations contribute to the outcome. This could also explain why we did not find any difference in cell viability *in vitro*.

In summary, we have infected a human mammary cell line with BLV *in vitro*. The new cell line does not release viral particles into the cell culture supernatant. Nevertheless, viral DNA was detected in the mammary epithelial cell genome. Previous studies indicate that the difference between BLV productive and non-productive infection is the efficiency in the reverse transcription [21]. In this case, we demonstrated that the virus is integrated, which indicates that the reverse transcription is not the critical step for BLV replication in this cell line. Moreover, it is well known that retrovirus expression can be repressed by epigenetic mechanisms both in natural and interspecies infections [22]. This could also explain the fact that no viral proteins were detected in MCF10A BLV cells supernatants.

More research needs to be done to elucidate if BLV plays a role in human breast cancer. The fact that we were able to detect a fragment of the viral gene *pol* in the MCF10A DNA is consistent with previous studies that detected other gene fragments by PCR and *in situ* PCR. Although the infection in MCF10A was not productive, we cannot disregard the possibility that this virus can play a role in human breast cancer.

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Conflicts of Interest

None.

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