Comparison of Two Quantitative Assays for Xenotropic Murine Leukemia Virus-Related Virus

Eiji SATO1)**, Rokusuke YOSHIIKAWA12 and Takayuki MIYAZAWA1)**

1)Laboratory of Signal Transduction, Department of Cell Biology, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawaharaacho, Sakyo-ku, Kyoto 606–8507, Japan
2)Graduate School of Human Environmental Studies, Kyoto University, Yoshida-Nihonmatsucho, Sakyo-ku, Kyoto 606–8501, Japan

(Received 1 August 2011/Accepted 16 September 2011/Published online in J-STAGE 30 September 2011)

ABSTRACT. Xenotropic murine leukemia virus-related virus (XMRV), a novel gammaretrovirus in humans, was found in patients with prostate cancer (PC) and chronic fatigue syndrome (CFS). However, there has been controversy whether XMRV is directly associated with human diseases. In this study, we developed a LacZ marker rescue assay using human embryonic kidney 293T cells and a focus assay using a feline fibroblastic sarcoma-positive leukemia-negative QN10S cells. XMRV induced prominent foci in QN10S cells and the viral titer determined by the focus assay was as high as that by the LacZ marker rescue assay. Because the focus assay is simple and sensitive, it will be useful for monitoring infectious XMRVs in CFS and PC patients and virological studies for XMRV.

KEY WORDS: focus assay, marker rescue assay, retrovirus, titration, XMRV.


A xenotropic murine leukemia virus (MLV)-related virus (XMRV) was firstly discovered in human patients with prostate cancer (PC) in the United States in 2006 [18]. In support of this finding, the other study examining XMRV prevalence in the United States described the positive detection of XMRV DNA in 6% of 334 prostate specimens of PC patients, and high grade PCs were more likely to show presence of XMRV than low grade ones [16]. Moreover, it was also documented that XMRV was detected in 32 (22%) of 144 PC specimens [2]. In 2009, Lombardi et al. reported that 68 of 101 (67.3%) patients with chronic fatigue syndrome (CFS) in the United States were infected with XMRV [9]. Following this report, Lo et al. detected MLV-related viral DNA sequences in 86.5% (32/37) of CFS patients [8]. The above results may suggest that XMRV is associated with PC or CFS; however, it is still unclear whether XMRV is directly associated with human diseases since many researchers failed to prove the presence of XMRV in PC or CFS patients in several countries [6]. Quite recently, it was proved that XMRV was generated by recombination of two defective endogenous retroviruses of nude mice during passage of a human prostate tumor xenograft in mice [12].

We have previously examined whether there was XMRV in blood of CFS patients as well as PC patients in Japan [4]. Consequently, we could not detect XMRV DNA in blood cells of CFS patients although plasma from two of 100 patients tested positive for the XMRV Gag antibody [4]. To quantitate infectious XMRV, Knouf et al. and Metzger et al. established a sarcoma-positive leukemia-negative (S+L–) assay that employed feline PG-4 cells, and also developed an alkaline phosphatase marker rescue assay using human HT-1080 cells [7, 10]. In this study, we also tried to establish reliable methods to detect infectious XMRV. In a previous report by Rodriguez and Goff [13], they found that XMRV did not grow efficiently in human embryonic kidney (HEK) 293T cells but grew efficiently in a human epithelial tumor cell line, LNCaP cells. However, the LNCaP cells are delicate; therefore, it is difficult to maintain the cells when they are cocultured with activated lymphocytes from patients in attempts to isolate XMRV. In this study, we examined the growth kinetics of XMRV in HEK293T cells and found that XMRV can grow efficiently in the cells. Then, we developed a marker rescue assay to titrate XMRV using HEK293T cells. Moreover, we developed a focus assay to detect and titrate XMRV using feline S+L– QN10S cells, and compared the sensitivity of the assays.

HEK 293T cells (ATCC, CRL-11268) cells [17] and QN10S cells (a feline fibroblast cell line) [5] were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA, U.S.A.). XMRV was prepared from the culture supernatant of HEK293T cells persistently infected with XMRV derived from 22Rv1 cells (ATCC: CRL-2505) [7]. Feline leukemia virus subgroup A (FeLV-A) was prepared from culture supernatant of HEK293 cells persistently infected with FeLV-A derived from an infectious molecular clone, pFGA-5 [11]. All stock viruses were filtrated through a 0.45 µm filter and kept at −80°C until used.

Firstly, we examined the growth kinetics of XMRV in
HEK293T cells by the LacZ marker rescue assay [15]. HEK293T cells transduced with nlsLacZ gene, termed 293T(LacZ) cells, were prepared as described previously [15]. The nlsLacZ gene has a nuclear localization signal upstream of the open reading frame of the lacZ gene [1]. HEK293T(LacZ) cells were seeded in 25 cm² tissue culture flasks at a concentration of 2.7 × 10⁶ cells. Infections were performed at 37°C by plating 2 ml of 18,000-fold diluted XMRV into target cells with 8 µg of polybrene (Sigma-Aldrich, St. Louis, MO, U.S.A.). Four hr post-infection, supernatants were replaced with fresh medium, and the infected cells were incubated for 30 days. Every three days, the culture supernatants were collected and immediately subjected to the LacZ assay as described previously [15]. Viral titers were determined by counting lacZ-positive blue foci, and expressed as infectious units (i.u.) per ml. As a result, XMRV grew gradually in 293T(LacZ) cells and the viral titer reached a plateau at 24 days post inoculation (d.p.i.) (Fig. 1). These data indicate that 293T(LacZ) cells can be applicable to the LacZ marker rescue assay to quantitate infectious XMRV.

Next, we titrate the stock virus of XMRV by the LacZ marker rescue assay using 293T(LacZ) cells. Cells were seeded in 25 cm² flasks one day prior to the virus inoculation. Then, 10-fold serially diluted samples of XMRV in 2 ml of medium were inoculated into cells in the presence of 8 µg/ml of polybrene in quadruplicate. The cells were incubated at 37°C for 4 hr for viral adsorption and the inoculum was then replaced with fresh medium. Every four days, the culture supernatants were collected and subjected to the LacZ marker rescue assay using HEK293T cells as target cells as described previously [14]. Briefly, culture supernatants from the flasks were filtrated through 0.45 µm filters, and then inoculated into HEK293T cells cultured in a 48-well-plate. Two days after inoculation, the cells were stained with X-gal, and the wells which had cells stained blue were judged as positive. Viral titers were expressed as 50% tissue culture infective dose (TCID₅₀) per ml. Consequently, the viral titer determined by the LacZ marker rescue assay reached the maximum titer (2.8 × 10⁶ TCID₅₀/ml) at 16 d.p.i. (Fig. 2).

Focus assays using S+L– cells have been used to detect infectious (replication-competent) gammaretroviruses [3]. A feline S+L– QN10S cells have been widely used to detect FeLV [5, 11] and a feline endogenous RD-114 virus [14]. Because XMRV is belonging to the genus gammaretroviruses [18], we applied the focus assay using QN10S cells to titration of XMRV. QN10S cells were seeded at a density of 2 × 10⁴ cells per well in 24-well plates one day before infection. Ten-fold serially diluted samples of XMRV in 500 µl of medium were inoculated into cells in triplicate in the presence of 8 µg/ml of polybrene. The cells were incubated at 37°C for 4 hr for viral adsorption and the inoculum was then replaced with 2 ml of fresh medium. At four days post-infection, prominent foci were observed in cells inoculated with XMRV (Fig. 3). At eight days post-infection, the viral titers were determined and expressed as focus-forming units (f.f.u.) per ml. The foci induced by XMRV were as large as those by FeLV-A (Fig. 3). The titer of the stock virus determined by the focus assay using QN10S cells was 6.0 ± 2.8 × 10⁶ f.f.u./ml and this value was nearly equivalent to that by the LacZ marker rescue assay (2.8 × 10⁶ TCID₅₀/ml).

Finally, we examined the growth kinetics of XMRV in HEK293T cells by the focus assay. XMRV was inoculated into 293T(LacZ) cells at a multiplicity of infection of...
0.00025 and cultured for 33 days. Every three days, the culture supernatants were collected and subjected to the focus assay. The viral titers were expressed as f.f.u. per ml. In HEK293T cells, the growth kinetics determined by the focus assay (Fig. 4) was parallel to that determined by the LacZ marker rescue assay (Fig. 1).

QN10S cells express the murine sarcoma virus (MSV) genome containing v-mos oncogene at a very low level. To induce transformed foci in QN10S cells, a sufficient amount of XMRV packaged with MSV genome should re-infect the cells in the vicinity of the initially infected cells. High transformation ability of QN10S cells may be explained by the assumption that the packaging efficacy of the MSV genome into XMRV virus particles was high, and the QN10S cells supported the replication of XMRV efficiently.

In the present study, we found that XMRV induced prominent foci in QN10S cells at 4 d.p.i. and the foci could be easily counted. Our focus assay appears to have similar sensitivity to that reported by Knouf et al., giving titers for XMRV of $2 \times 10^6$ to $10^7$ f.f.u./ml [7]. The focus assay using QN10S cells was as sensitive as the LacZ marker rescue assay using HEK293T cells; therefore, the assay will be useful for monitoring a small amount of infectious XMRV in CFS and PC patients and virological studies for XMRV.

ACKNOWLEDGMENTS. We are grateful to Prof. Os Jarrett (Glasgow University, Glasgow, U.K.) for providing QN10S cells. This study was partly supported by grant-in-aid from the Bio-oriented Technology Research Advancement Institution.

REFERENCES


