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Establishment of cell lines latently infected with non-oncogenic murine gammaherpesvirus 76

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Summary. – Murine gammaherpesviruses 68 (MHV-68) and 78 (MHV-78), both inducing tumors in mice and a latent infection in cells *in vitro*, serve as models for study of human oncogenic gammaherpesviruses, namely Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). In this work, we succeeded in establishing a latent infection of HeLa and CGL1 cell lines with non-oncogenic murine gammaherpesvirus 76 (MHV-76), which differs from MHV-68 and MHV-78 besides by oncogenicity also by deletion of M1-M4 genes and eight tRNA-like sequences. Viral latency in these cell lines, λHeLa and λCGL1, was demonstrated by the presence of viral DNA, suppression of viral latency-associated ORF73 gene and appearance of low amounts of infectious virus following treatment with phorbol 12-myristate 13-acetate (PMA). Both latently infected cell lines showed irregular presence of viral antigen originating apparently from spontaneous reactivation. The growth of latently infected cells in culture was similar to that of non-infected ones. The latently-infected λHeLa cells did not induce tumors in mice following subcutaneous inoculation. These results (i) confirm that MHV-76 is the only non-oncogenic murine gammaherpesvirus of all the so far tested ones, (ii) suggest that some of the genes deleted in MHV-76 might be responsible for the oncogenicity of murine gammaherpesviruses, (iii) confirm that viral ORF73 is one of major latency-associated genes that is suppressed during virus reactivation, and (iv) present MHV-76 as another murine gammaherpesvirus useful as a model for study of gammaherpesvirus pathogenesis, oncogenicity, latency and reactivation.

Keywords: murine gammaherpesvirus; latent infection; reactivation; oncogenicity; ORF73

Introduction

The subfamily *Gammaherpesvirinae* of the family *Herpesviridae* in the order *Herpesvirales* includes human and animal lymphotropic herpesviruses, among them EBV, KSHV, and MHV-68 (Davison *et al*., 2009). The lattter virus, isolated from a small rodent *Myodes glareolus* in 1976 in Slovakia (Blaškovič *et al.,* 1980), was classified to a new species, *Murid herpesvirus 4* in the *Rhadinovirus* genus (Murphy *et al*., 1995). Similar viruses, isolated at the same time or later from various small rodents (MHV-60, MHV-72, MHV-76, MHV-78, MHV-Šumava, MHV-4556, and MHV-5682), remain so far unclassified (Blaškovič *et al*., 1980; Mistríková and Blaškovič, 1985; Kožuch *et al*., 1993). They are considered as murine gammaherpesviruses more or less differing from MHV-68 in genetical, pathogenetical and immunological properties (Mistríková *et al*., 1985, 1996, 2004; Kožuch *et al*., 1993; Oda *et al*., 2005; Blaškovičová *et al.,* 2007). Analysis of gene expression in MHV-68 revealed that K3, RTA, M8, and DNA polymerase genes are expressed in lytic phase, while M2, M3, M9, M11, ORF73, and ORF74 genes are expressed during latency (Rochford *et al*., 2001). ORF73 encodes the latency-associated nuclear antigen (LANA), which plays roles in replication of latent viral episome and maintenance of latency in spleen fol-

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Abbreviations: EBV = Epstein-Barr virus; KSHV = Kaposi's sarcoma-associated herpesvirus; IFA = immunofluorescence assay; PMA = phorbol 12-myristate 13-acetate; DMSO = dimethylsulfoxid; LANA = latency-associated nuclear antigen; MHV-60, MHV-72, MHV-68, MHV-76, MHV-78, MHV-Šumava, MHV-4556, MHV-5682 = murine gammaherpesviruses 60, 68, 76, 78, Šumava, 4556, and 5682, respectively

lowing intranasal (i.n.) infection (Virgin *et al*., 1997; Marques *et al*., 2003; Moorman *et al*., 2003; Fowler *et al*., 2003).

The ability to induce tumors in mice is a very important but differently expressed feature of most murine gammaherpesviruses as demonstrated by various percentage of inoculated mice which developed tumors: 7% (MHV-78), 9% (MHV-68), 11% (MHV-72), 14.5% (MHV-Šumava), and 22% (MHV-60) (Mrmusová-Šupolíková *et al*., 2003; Sunil-Chandra *et al*., 1994; Mistríková *et al*., 1996, 2002; Pappová *et al*., 2004). On the other hand, MHV-76 did not exert any oncogenicity in mice (Chalupková *et al.,* 2008). Virus-induced tumors could be used for derivation of cell lines harboring viral genome and representing a latent viral infection, as shown by S11 and NB-78 cell lines carrying MHV-68 and MHV-78, respectively (Usherwood *et al.,* 1996; Mistríková *et al.,* 2006). The course of MHV-76 infection in mice showed a route-dependent manner; as this virus induced latency in the spleen after intraperitoneal but not intranasal inoculation. In contrast, both routes of infection led to latency with MHV-68 (Macrae *et al.,* 2001; Clambey *et al*., 2002).

In this work, we attempted (i) to establish cell lines latently infected with MHV-76 differing from other murine gammaherpesviruses in several important properties, (ii) characterize them regarding phenotype, growth properties, latency and virus reactivation, and (iii) examine their possible tumorigenicity.

Materials and Methods

Viruses. MHV-68 and MHV-76 stocks were prepared in Vero cells by infection at MI of 1, harvesting at 72–96 hrs p.i., sonication, clarification by low-speed centrifugation, and storage at -70°C.

Cells. HeLa and non-tumorigenic HeLa-human skin fibroblast hybrid CGL1 cells (Stanbridge, 1976) as well as latently MHV-76-infected λHeLa, λCGL1, and SP2/0 cells and thymocytes were grown in DMEM with 7% FCS and antibiotics. Thymocytes explanted from thymuses of BALB/c mice, mouse myeloma SP2/0 cell line, and NB-78 cell line were prepared or cultured as described (Horibata and Harris, 1970; Mistríková *et al.,* 2006).

Mice. BALB/c mice were supplied by the Faculty of Veterinary Medicine, Brno, Czech Republic.

Experiments on mice. A group of 17 newborn mice was inoculated subcutaneously in cervical area with 10^5 λHeLa cells per mouse in 20 µl. A control group of 10 newborne mice was inoculated similarly with HeLa cells. The mice were killed 30 days p.i. by cervical dislocation under ether anesthesia and their lungs and spleens were removed, homogenized and stored at -70°C until use.

Virus titration. Frozen homogenates of organs were thawed, clarified by low-speed centrifugation, diluted in DMEM supplemented with 2% of heat-inactivated bovine serum, 300 µg/ml glutamine, and 80 µg/ml gentamicin, and titrated on Vero cell monolayer cultures. CPE was read after 3–7 days of incubation at 37°C in 5% CO_2 and hematoxylin-eosin staining. Virus infectivity titers were expressed in $TCID_{50}$/ml.

Immunofluorescence assay (IFA) of viral particle antigen was done by an indirect method. Briefly, cells in suspension were stained with a polyclonal rabbit antibody to MHV-68 followed by a FITC-conjugated anti-rabbit antibody (ALEXA 488, Molecular Probes, USA). Washing with PBS was included in between individual steps. Finally, cells were mounted and examined under fluorescence microscope. The percentage of positive cells was calculated.

PCR for viral DNA was carried out as described.Total DNA was isolated from tissues or cells using the DNeasy Tissue Kit (Qiagen) according to the manufacturer´s instructions. The reaction mixture based on the GoTaq Green Master Mix (Promega) contained total DNA, and the primers specific for MHV-68 ORF4 gene 5'-CAAATGCCCACACTAACATCTCCTAAA-3' (forward) and 5'-AGTATATAGGCTGGGGACTTGACTTCG-3' (reverse) (Sigma-Aldrich). Positive control contained MHV-68 DNA, while negative one lacked template DNA. The reaction consisted of initial denaturation at 84°C for 3 mins, 40 cycles of 94°C/1 min, 65°C/1 min, and 72°C/1 min, and final extension at 72°C for 3 mins. PCR products were analyzed by electrophoresis in 1.2% agarose gels and ethidium bromide staining. The expected size of specific PCR product was 410 bp.

Reactivation of latent virus. PMA was added to one-day-old cultures of latently infected cells to final concentration of 20 ng/ml. After 24 hrs, cells were harvested and used for assaying viral ORF73 gene expression and infectious virus.

RT-PCR for viral ORF73 gene transcripts was carried out as followed. Total RNA was isolated using the Instapure System (Eurogentec, Belgium), dissolved in DEPC-treated water and reverse-transcribed with M-MuLV reverse transcriptase (Finnzymes OY) using random heptanucleotide primers. The reaction mixture for a nested PCR contained the GoTaq Green Master Mix, 5%DMSO and primers. Both PCRs consisted of initial denaturation at 95°C for 5 mins, 35 cycles of 95°C/45 secs, 59°C/40 secs, and 72°C/30 secs, and final extension at 72°C for 5 mins. Outer primers 5'-TAG TACCTTCTACGCTGCTC-3' (forward) and 5'-GTAGGTGCT TCAACAAACCA-3' (reverse), and inner primers 5'-CCAA GGGTAAACAGCTAACT-3' (forward) and 5'-CCTTCATCA CCAGTACATGAG-3' (reverse) were employed. A positive control consisted of MHV-68 DNA, while a negative one lacked template DNA. Final PCR products were analyzed by electrophoresis in 1.2% agarose gels and ethidium bromide staining. The expected size of specific PCR product was 363 bp.

Results

Establishment and characterization of latently infected cell lines

Using infection with MHV-76 at MI of 0,01 and frequent passaging of infected cells in standard culture medium two latently infected cell lines were established, namely λCGL1 and λHeLa. Use of higher MI resulted in cell destruction due to viral CPE and the inability to undergo passaging (data not shown). Cytological investigation revealed a changed

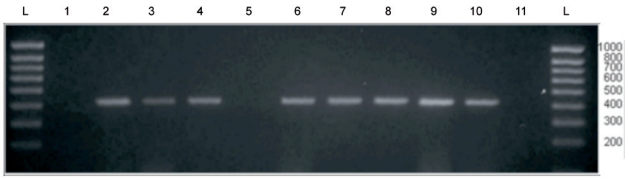

Fig. 1

Detection of viral DNA (ORF4 gene) in latently infected cell lines

CGL1 cells (lane 1);. λCGL1 cells , passages 21 (lane 2), 34 (lane 3), and 72 (lane 4); HeLa cells (lane 5); λHeLa cells, passages 24 (lane 6), 38 (lane 7), 56 (lane 8), and 83 (lane 9); MHV-68 DNA, positive control (lane 10); PCR without template DNA, negative control (lane 11); DNA ladder (lane L).

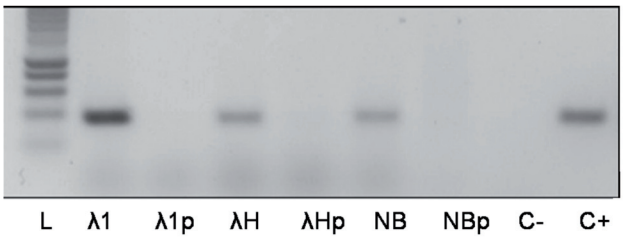

Fig. 2

Suppression of viral ORF73 gene in latently infected cell lines following PMA treatment

The RT-PCR product consisted of 363 bp. DNA ladder (lane L); λCGL1 cells (lane λ1); CGL1 cells treated with PMA (lane λ1p); λHeLa cells (lane λH); λHeLa cells treated with PMA (lane λHp); NB-78 cells (lane NB); NB-78 cells treated with PMA (lane NBp); PCR with no template DNA, negative control (lane C-); PCR with MHV-68 DNA, positive control (lane C+).

phenotype of latently infected cells and inability to form continuous monolayer (data not shown). Viral DNA was demonstrated by PCR regularly in passages of both latently infected cell lines (Fig. 1).

Viral latency-associated ORF73 gene was expresed in both latently infected cells lines (Fig. 2). Treatment of the latently infected cells with PMA, an agent causing virus reactivation, resulted in suppression of this gene and appearance of infectious virus in the medium (Table 1). A similar effect of PMA could be also seen in NB-78 cells latently infected with MHV-78.

Viral particle antigen indicating spontaneous virus reactivation was observed irregularly in passages of both latently infected cell lines (data not shown).

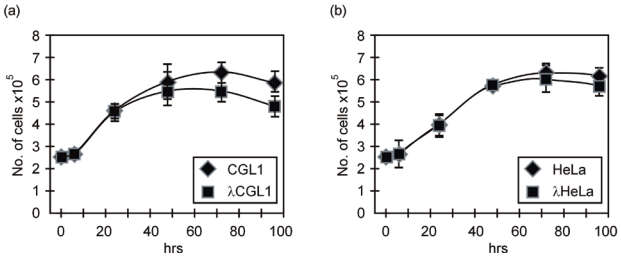

Fig. 3

Effect of latent MHV-76 infection on the growth of Lambda CGL1 and Lambda HeLa cells

Analysis of cell growth in culture for 100 hrs revealed no differences between the latently infected λCGL1 and λHeLa cells and non-infected parental CGL1 and HeLa cells, respectively (Fig. 3). SP2/0 cells latently infected with MHV-76 or MHV-68, used for comparison, also did not differ from their non-infected counterparts in the growth during 47 passages (Fig. 4). On the other hand, a latent MHV-76 infection positively affected the growth of thymocytes in culture as they reached 17 passages compared to 11 passages of non-infected thymocytes. In contrast, a latent MHV-68 infection did not have any effect on the growth of thymocytes in culture (Fig. 5).

Using a similar approach a latent MHV-76 infection was also established in SP2/0 cell line and cultured thymocytes (data not shown).

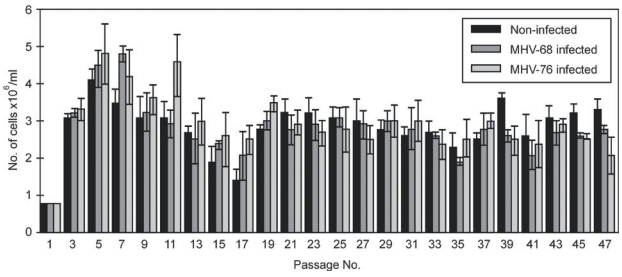

Fig. 4

Effect of latent MHV-76 or MHV-68 infection on the growth of SP2/0 cells

Final cell count in individual passages was estimated.

Table 1. Infectious virus produced by latently infected cells following PMA treatment

Cells					
λCGL1	λCGL1+PMA	λHeLa	λHeLa+PMA	NB-78	NB-78+PMA
–	+	–	++	–	+

Infectious virus absent (–) or present in undiluted (+) or 10^{-1}diluted (++) culture medium, respectively.

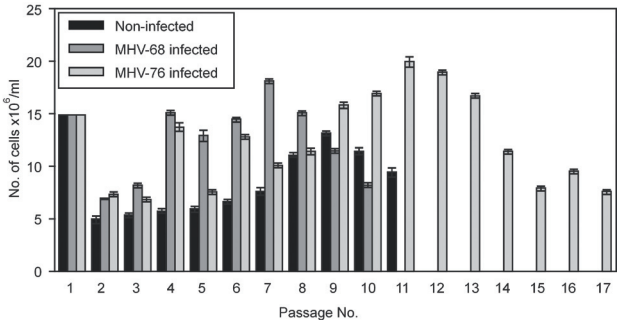

Fig. 5

Effect of latent MHV-76 or MHV-68 infection on the growth of thymocytes

Non-tumorigenicity of λHeLa cells

Inoculation of λHeLa cells carrying MHV-76 genome into mice did not lead to development of tumors in the inoculation area. We could not detect either viral DNA or infectious virus in the spleen and lungs or viral antigen in the splenocytes of these mice (data not shown).

Discussion

Latently infected cell lines provide useful system for study of latency and molecular characteristics of viral infection. A very common way how to obtain such a system is derivation of cell lines from tumor tissues. In general, viral genome can be present in these cell lines or not. For example, there are EBV genome-negative cell lines such as BJA-B, U-698M, and U-715M (Pagano *et al.,* 1973; Klein *et al.,* 1974; Nilsson *et al.,* 1974; Menézes *et al.,* 1975).

Also tumors originating from mice infected with some murine gammaherpesviruses have been used for derivation of cell lines. So Usherwood *et al.* (1996) obtained from tumors of mice infected with MHV-68 five cell lines, of which only one, S11 was viral genome-positive, while four were negative. Using MHV-78 and a similar approach Mistríková *et al.* (2006) obtained a single cell line, NB-78, which was viral genome-positive.

Since MHV-76 infection did not lead to tumor development in mice (Chalupková *et al.*, 2008), we had to employ another way of obtaining a cell line carrying MHV-76 genome. It was infection of selected cell lines, CGL1 and HeLa, at a low multiplicity (0.01 $TCID_{50}$/cell) and their regular passaging that led to latently infected cell lines λCGL1 and λHeLa. Both of them showed a changed phenotype and were continuously MHV-76 genome-positive.

The analysis of cell growth in culture revealed that the latent infection with MHV-76 did not influence CGL1 and HeLa cell lines in this regard. Since MHV-76 is a lymphotropic virus, we also investigated its effect on the growth of thymocytes in culture. We found that the growth and survival of thymocytes latently infected with MHV-76 were markedly stimulated. Likewise, Steinitz *et al.* (1975) demonstrated a stronger proliferation of EBV genome-positive compared to EBV genome-negative cells.

As for the potential oncogenicity of MHV-76, we showed here that inoculation of λHeLa cells into mice did not lead to tumor development. In contrast, NB-78 cells carrying MHV-78 we shown to induce tumors under the same conditions (Mrmusová-Šupolíková *et al.*, 2003). In the study of Daibata *et al.* (1996), EBV-positive cell lines derived from mantle cell lymphoma induced tumors in nude mice, while a lymphoblastoid B-cell line, established by infecting normal B-lymphocytes *in vitro* with EBV, did not. All these results indicate that besides possible differences between viruses also those between cells containing virus or carrying viral genome might contribute to viral oncogenicity. As for the oncogenicity of various murine gammaherpesviruses, all the so far obtained results support the view that MHV-76 is the only non-oncogenic one.

Since we did not detect viral DNA or infectious virus in the spleen and lungs or viral antigen in the splenocytes of the mice inoculated with λHeLa cells, we assume that there was neither productive nor latent infection with possible virus reactivation.

Summing up, we established two cell lines latently infected with MHV-76, characterized them, and confirmed non-oncogenicity of MHV-76 and the essential role of ORF73 gene in maintenance of latency of murine gammaherpesviruses.

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