Acta virologica 54: 55 – 60, 2010 doi:10.4149/av_2010_01_55

Tumors induced by Murine herpesvirus 60 or by cell line NB-78 derived from a tumor induced by Murine herpesvirus 78 show presence of the inducing viruses

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Received November 2, 2009; accepted November 20, 2009

Summary. – Murine herpesviruses 60 and 78 (MHV-60, MHV-78), closely related to Mouse herpesvirus strain 68 (MHV-68), are oncogenic lymphotropic gammaherpesviruses, which may serve as models for study of human oncogenic gammaherpesviruses such as Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). In this work, we attempted to detect an analog of the MHV-68 ORF73 gene in tumors induced in mice either directly by MHV-60 or indirectly by MHV-78 via inoculation of NB-78 cells derived from a tumor induced by MHV-78. Besides the ORF73 gene, viral antigen and infectious virus were searched for. We succeeded in inducing lymphomas in mice by NB-78 cells and thus confirmed their transformed character. Importantly, we showed that the tumors induced by either MHV-60 or NB-78 cells were positive for the ORF73 gene, viral antigen and infectious virus. These results confirmed the generally accepted hypothesis about the connection between reactivation of latent gammaherpesviruses and malignant tumorigenesis.

Keywords: murine gammaherpesviruses; ORF73 gene; NB-78 cells; mice, tumors

Introduction

Herpesviruses are known for their ability to establish life-long latent infection. The interest in the biology of the gammaherpesviruses stems largely from their association with a variety of malignancies. EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease and KSHV with Kaposi sarcoma and AIDS-related B-cell lymphomas. Studying the biological and pathogenetic characteristics of EBV or KSHV is limited by their strict host range. The MHV-68 infection of laboratory mice represents a small animal model for the study of gammaherpesvirus pathogenesis (Blaškovič *et al*., 1980).

MHV-68 has been classified to the *Murid herpesvirus 4* species, the *Rhadinovirus* genus, and the *Gammaherpesvirinae* subfamily (Fauquet *et al.,* 2005), while MHV-60 and MHV-78 were not yet classified but, based on their very close relation to MHV-68, are asssumed also to represent gammaherpesviruses.

MHV-68 and MHV-60, both isolated from *Myodes glareolus,* induced tumors in 9% and 22% of mice, respectively, while MHV-78, isolated from *Apodemus flavicollis*, induced tumors in 7% of mice (Blaškovič *et al.*, 1980; Sunil-Chandra *et al*., 1994; Pappová *et al.,* 2004; Mrmusová-Šupolíková *et al.,* 2003). The NB-78 cell line, derived from a tumor induced in mice by MHV-78 after 252 days-long infection, showed the presence of selected viral genes, viral antigen (in 1–2% cells) and infectious virus (in the passages 43–45 from 100 so far done) (Mrmusová-Šupolíková *et al.,* 2004; Mistríková *et al.,* 2006).

It seems that all the so far tested murine gammaherpesviruses (MHV-68, MHV-60, MHV-72, MHV-76, MHV-78,

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Abbreviations: EBV = Epstein-Barr virus; EBNA = Epstein-Barr nuclear antigen; KSHV = Kaposi's sarcoma-associated herpesvirus; MHV-60 = Murine gammaherpesvirus 60, MHV-68 = Mouse herpesvirus strain 68; MHV-78 = Murine gammaherpesvirus 78; LANA = latent nuclear antigen; IFA = immunofluorescence assay; p.i. = post infection/inoculation

and MHV-Šumava) except the deletion mutant of MHV-76 lacking M1-M4 genes and other sequences (Macrae *et al.,* 2001) are involved in malignant neoplasm development in a lymphoproliferative disease in mice similar to that caused by EBV in humans.

MHV-68 established a latent infection in B-lymphocytes following an acute respiratory infection in mice (Sunil-Chandra *et al*., 1994). MHV-68 DNA was detected in alveolar epithelial cells of lungs, macrophages, trafficking B-lymphocytes carrying the genome to the spleen and lymph nodes, NK cells and dendritic cells (Stewart *et al.,* 1998).

The mechanism by which B-cells, mononuclear leukocytes and dendritic cells become latent carriers of MHV-68 DNA is still obscure. Analysis of this latency showed its similarity to that of EBV, which is associated with the expression of Epstein-Barr nuclear antigen 1 (EBNA1), a kind of latency-associated nuclear antigen (LANA) (Kieff and Rickinson, 2001). During the MHV-68 latency, B-lymphocytes carrying viral DNA express a LANA encoded by the ORF73 gene (Fowler *et al.,* 2003). MHV-68 LANA is logicaly considered a homolog of KSHV LANA1 (Starp and Boskoff, 2000) and EBV EBNA1, all functioning in the replication and maintanance of latent viral genome (Rajčáni and Kúdelová, 2007). Since gammaherpesviruses establish a life-long latency in the host and are associated with a number of malignant diseases, they particularly provide an excellent model for studying viral latent genome and its association with tumorigenesis.

This study was focused on detection of the ORF73 gene, viral antigen and infectious virus in tumors induced in mice either directly by MHV-60 or indirectly by MHV-78 via inoculation of NB-78 cells derived from a tumor induced by MHV-78.

Materials and Methods

Virus. A MHV-60 stock was prepared by infecting Vero cells and harvesting them 72–96 hrs post infection (p.i.). The cells were sonicated, clarified by low-speed centrigugation, and stored at -70°C until use. Infectious virus was assayed by plaque titration in Vero cells.

Cells. The NB-78 cell line, derived from a lymphoma of a BALB/c mouse infected with MHV-78, was employed (Mistríková *et al.,* 2006). This cell line shows an epitheloid transformed morphology (Fig. 1) and a diploid complement of 40 chromosomes. The cells were cultivated in DMEM supplemented with 7% of FBS, 300 μg/ml glutamine, and 80 μg/ml gentamicin.

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

Experiments on mice. A group of twenty 6-week-old mice was infected intranasally with 2 x 10^4 PFU of MHV-60 per mouse in 20 μl under ether anaesthesia. Another group of seventeen 4-day-old newborne mice was inoculated subcutaneously in cervical area with 10^5 NB-78 cells per mouse in 20 μl. The mice were killed 18 months p.i. (the MHV-60 group) or 21 days p.i. (the NB-78 group) by cervical dislocation under ether anaesthesia. The tumors and selected organs (lungs, spleen) were removed, homogenized and stored at -70° C until use. The cells from tumors induced by MHV-60 were cultivated *in vitro* for over 30 days. Blood samples were taken from *sinus orbitalis* and heparinized. Differential white blood cell count was carried out in standard manner.

Virus titration. Infectious virus titers were assayed on VERO cells. Frozen homogenates of tumors or 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 in VERO cell monolayer cultures. CPE was read after 3–7 days of incubation at 37°C in 5% CO_2 and staining .

Immunofluorescence assay (IFA). Viral antigen was assayed by an indirect IFA as follows. Cells in suspension (100 μl) were washed with PBS, stained with a polyclonal rabbit antibody to MHV-68 (dilution 1:100) at 37°C for 45 mins, washed with PBS, stained with rhodamine-conjugated goat anti-rabbit IgG (H + L) (1:10 dilution, Immunotech, Slovak Republic) at 37°C for 45 mins, washed with PBS, mounted, and examined under fluorescence microscope. The percentage of cells positive for fluorescence was calculated.

PCR. The ORF73 gene of viral DNA was assayed by a nested PCR. Total DNA was isolated using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. The reaction mixture consisted of template DNA, the GoTaqGreen MasterMix (Promega), and the following primer pairs, designed originally for MHV-68 ORF73 gene and purchased from Sigma-Aldrich. In the first PCR, the primers 5'-TAGTACCTTCTACGCTGCTC-3' (forward) and 5'-GTAGGTGCTTCAACAAACCA-3' (reverse) were used, while in the 2nd PCR the primers 5'-CCAAGGGTAAACAGCTAACT-3' (forward) and 5'-CCTTCATCACCAGTACATGAG-3' (reverse) were employed. The use of these primers for detection of ORF73 genes of MHV-60 and MHV-78 was justified by the finding of the identity of the amplified part of sequence of ORF73 gene of MHV-68, MHV-60 and MHV-78 (Z. Hrabovská, unpublished data). Negative control lacked template DNA, while positive control contained MHV-68 DNA. 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. Final PCR products were analyzed by electrophoresis in 1.2% agarose gels. The size of the amplified ORF73 gene fragment was 363 bp.

Results

Induction of tumors in mice by MHV-60 and NB-78 cells

In the group of mice infected with MHV-60, four tumors were found 18 months p.i. in the lungs, kidneys and lymphatic nodes in the colon area. In the group of mice inoculated with NB-78 cells, also four tumors were observed 21 days p.i. in the area of inoculation (Fig. 2). Differential white blood cell count of the MHV-60-infected mice with tumors showed from 4% to 7% of atypical leukocytes, while that of the tumor mice inoculated with NB-78 cells displayed an essentially similar range, from 2% to 6% of atypical leukocytes. Generally, the percentage of atypical leukocytes in the infected mice varied from 4% to 20%.

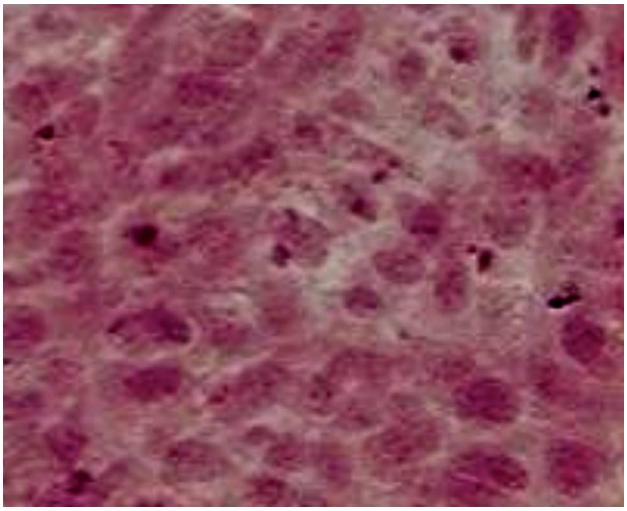

Fig. 1

Morphology of NB-78 cells

The 50th cell passage, hematoxylin-eosin staining.

Fig. 2

Mice with tumors induced by NB-78 cells

Virus presence in tumors induced by MHV-60

The ORF 73 gene was present in all four tumors as well as in the spleen and the lungs (Fig. 3, Table 1). The cells from tumors kept in culture also showed presence of the ORF73 gene when tested on days 7 and 20 of cultivation. The frequency of cells positive for viral antigen was 5–10% for the tumors as well as the spleen (Fig. 4b). All four tumors showed higher infectious virus titers compared with the spleen and lungs from the same mice. In comparing positivities for the ORF73 gene, viral antigen and infectious virus titer, the first two parameters were weaker compared with the last one.

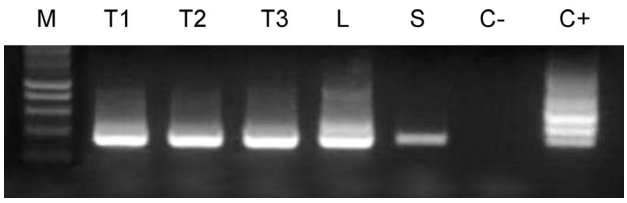

Fig. 3

Detection of MHV-60 ORF73 gene in tumors induced by MHV-60 and in organs of tumor-bearing mice

Agarose gel electrophoresis of PCR products. DNA size markers (M), tumors (T1-T3), spleen (S), lungs (L), negative control (C^-), positive control (C^+), tumors (T1-T3), lungs (L), spleen (S).

Virus presence in tumors induced by NB-78 cells

The ORF 73 gene was present in all four tumors as well as in the spleen and the lungs (Fig. 5, Table 2). Parallel experiments showed the presence of the ORF73 gene also in NB-78 cells (data not shown). All four tumors showed higher infectious virus titers compared with the spleen and lungs from the same mice. The frequency of cells positive for viral antigen was 5–10% for the tumors as well as the spleen (Fig. 4a). The comparison of the three tested viral

Table 1. Detection of MHV-60 in tumors induced by MHV-60 and in organs of tumor-bearing mice

Viral marker	Tumor/organ				
	Tumor 1	Tumor 2	Tumor 3	Lungs	Spleen
ORF73 gene[1]	+	+	+	+	+
Viral antigen[1]	+	+	+	ND	+
Infectious virus[2]	(+++)	(+++)	(+++)	(++)	(++)

[1]Positivity (+), ND = not done. [2]Positivity only in concentrated form (+), also in 1:10 dilution (++), also in 1:100 dilution (+++).

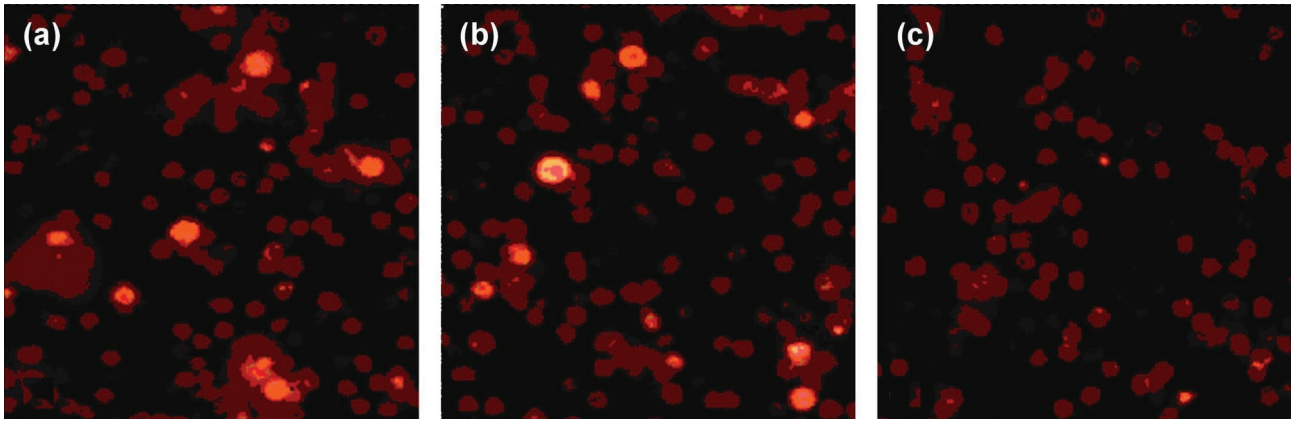

Fig. 4

Viral antigen in splenocytes of tumor-bearing mice inoculated with Nb -78 cells (a) or infected with MHV-60 (b)

Immunofluorescence assay. Negative control (c^-).

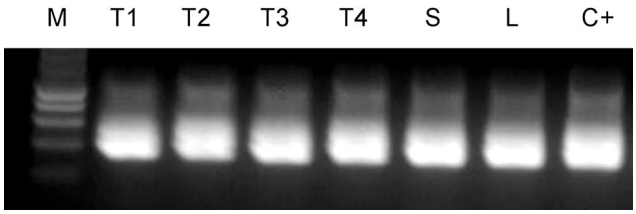

Fig. 5

Detection of MHV-78 ORF73 gene in tumors induced by NB-78 cells and in organs of tumor-bearing mice

Agarose gel electrophoresis of PCR products. DNA size markers (M), tumors (T1-T4), spleen (S), lungs (L), positive control (C^+).

parameters gave a result similar to that obtained with MHV-60-induced tumors.

Discussion

The ability of MHV-68 to induce a persistent infection was proven for the first time by explantation of the lungs, spleen, and kidneys from surviving mice after several months p.i. (Rajčáni *et al.,* 1985). The persistence of MHV-68 in B-lymphocytes was characterized as a non-productive latency (Sunil-Chandra *et al.,* 1993). Tumorigenesis has been proven for all so far tested murine gammaherpesviruses except the MHV-76 deletion mutant lacking M1-M4 genes and other sequences (Macrae *et al.,* 2001).

This work describes a search for virus presence in tumors induced by two different murine gammaherpesviruses, MHV-60 or MHV-78.

In case of MHV-60, the infection of mice with this virus was characteristic by a shorter early acute phase compared with other murine gammaherpesviruses. Four of 20 infected mice developed tumors 18 months p.i. in accord with a previous finding of 20% frequency by Pappová *et al.* (2004) and with a general view that MHV-60 is the most oncogenic of the so far isolated murine gammaherpesviruses. We succeeded in detecting viral DNA, in particular viral ORF73 gene, in all the tumors as well as in the spleen and the lungs of tumor-bearing mice. The ORF73 gene was chosen as a conserved gammaherpesvirus gene, which encodes a LANA. The latter protein was expressed in B-lymphocytes carrying MHV-68 DNA during the latency (Virgin *et al.,* 1997; Fowler *et al.,* 2003). The detection of viral DNA in tumors induced by MHV-60 is the first report of such kind

Table 2. Detection of MHV-78 in tumors induced by NB-78 and in organs of tumor-bearing mice

Viral marker	Tumor/organ					
	Tumor 1	Tumor 2	Tumor 3	Tumor 4	Spleen	Lungs
ORF73 gene[1]	+	+	+	+	+	+
Viral antigen[1]	+	+	+	+	+	ND
Infectious virus[2]	(+++)	(+++)	(+++)	(++)	(+)	(+)

For the footnote see Table 1.

on gammaherpesviruses. In our experiments, the presence of viral DNA corresponded to that of viral antigen and infectious virus, indicating the presence of a latent but reactivable viral genome. The presence of infectious virus in the spleen and the lungs in particular demonstrated such a reactivation and spread to these organs.

In case of MHV-78, tumors were induced by NB-78 cells, derived from a lymphoma induced by MHV-78. NB-78 cells were characterized until now as containing M1-M4, ORF4 and ORF50 genes of MHV-78 (Mistríková *et al.,* 2006; Polčicová *et al.,* 2008). In this study, also the ORF73 gene was proven. NB-78 cells induced massive lymphomas in 23.5% of mice, while MHV-78 alone induced malignancies in 7% of mice only (Mrmusová-Šupolíková *et al.,* 2003). Viral ORF73 gene was detected in all tumors and selected organs of tumor-bearing mice. Again, viral DNA corresponded to viral antigen and infectious virus.

The positivity of the spleen and lungs for the viral markers tested was similar to that seen in MHV-60-infected mice.

The capability of a tumor cell line carrying MHV-68 DNA to induce malignancies was demonstrated by Usherwood *et al.* (1996). Interestingly, in their work, only one of five tumors induced by MHV-68 carried viral DNA, while in our study, all four tumors induced by NB-78 cells carried viral DNA.

As mentioned above, MHV is used as a murine model of human gammaherpesviruses such as EBV and KSHV. There are several cell lines carrying EBV genome. Two of them, HONE-1 and HNE-1, both derived from nasopharyngeal carcinomas, were examined for presence of viral DNA as well as EBNA. EBV genome was detected up to the passages 40 and 45 in HONE-1 and HNE-1 cells, respectively. EBNA was detected only in 10% of HNE-1 cells but in 85–90% of HONE-1 clone 40 cells (Glaser *et al.,* 1989). This variation corresponds to the results of search for EBV-associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines carrying EBV genome, which showed that only a minority (below 5%) of the producer cells were antigen-positive, while all the non-producer cells were negative. Besides, the positivity varied in dependence on cell clone (Reedman *et al.,* 1973). An EBV-genome negative B-lymphocyte cell line derived from Burkitt lymphoma produced malignant tumors in thymus-deficient nude mice (Klein *et al.,* 1975). These findings are in accord with those obtained by us in this study.

Summing up, using two different murine gammaherpesviruses, MHV-60 and MHV-78, we demonstrated viral origin of solid tumors in BALB/c mice and confirmed *in vivo* a transformed phenotype of NB-78 cells.

Acknowledgement. This work was supported by the grant No. 1/0083/08 from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences, and APVV 51-005-005.

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