ESTABLISHMENT AND CHARACTERIZATION OF A TUMOR CELL LINE DERIVED FROM A MOUSE INFECTED WITH MURINE GAMMAHERPESVIRUS 78

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Summary. – A tumor cell line, NB-78, was derived from a lymphoma from a BALB/c mouse infected with Murine gammaherpesvirus 78 (MHV-78). Cultures of the cells of this line underwent till now more than 100 passages**,** displaying an epiteloid transformed morphology and a diploid complement of 40 chromosomes. Viral antigen was detected in 1–2% of cells by immunofluorescence (IF) test. Reactivation of latent MHV-78 was proved by detecting infectious virus in culture medium only at passages 43–45. The presence of viral M1, M2, M3, and M4 gene sequences in the genome of the cells was demonstrated by PCR. NB-78 is the first continuous cell line, which originates from a tumor of a MHV-78-infected host, harbors viral genome or at least its several genes, and produces infectious virus only rarely upon reactivation. It can be assumed that this cell line is primarily associated with MHV-78 and will serve as an invaluable tool for studying the MHV-78 latency.

Key words: tumor cell line; BALB/c mice; MHV-78; karyotyp; morphology; MHV-genes

Introduction

MHV-78 is one of several isolates belonging to the species *Murid herpesvirus 4* (the genus *Rhadinovirus,* the subfamily *Gammaherpesvirinae* (Fauquet *et al*., 2005). These isolates are closely related, but they differ in some biological properties (Svobodová *et al*., 1982a,b). MHV-78 was isolated from *Apodemus flavicollis* mice*,* while another member of this virus species, Murine gammaherpesvirus 68 (MHV-68) originated from another murine species, *Clethrionomys glareolus* (Blaškovič *et al*., 1980).

MHV-68, the first and most thoroughly studied murine gammaherpesvirus, and the mouse represent amenable, small animal model for the study of gammaherpesvirus pathogenesis (Efstathiou *et al*., 1990a,b; Simas *et al*., 1998). MHV-68 and MHV-78 in comparison with other strains lacked a polypeptide with apparent molecular weight of 46 K (Reichel *et al*., 1991).

MHV-68 established a latent infection in B-lymphocytes, following an acute respiratory infection (Sunil-Chandra *et al*., 1994). At late stages of long-term infection, 10% of mice developed a lymphoproliferative disease. A high proportion (~50%) of these mice displayed high-grade lymphomas associated with the spleen or cervical mesenteric lymph nodes (Sunil-Chandra *et al*., 1994; Mistríková *et al*., 1996, 1999). Lymphoprolipherative diseases were not only lymphomas, but leukemia-like syndromes too (Mistríková and Mrmusová, 1998; Mistríková *et al*., 2004).

Both MHV-68 and MHV-78 have an oncogenic potential, inducing tumors in 11% and 7% of mice, respectively (Sunil-Chandra *et al*., 1994; Mrmusová-Šupolíková *et al*., 2004).

Regarding the cell transformation, the most thoroughly studied gammaherpesvirus is Epstein-Barr virus (EBV).

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Abbreviations: EBV = Epstein-Barr virus; IF = immunofluorescence; MHV-68 = Murine gammaherpesvirus 68; MHV-72 = Murine gammaherpesvirus 72; MHV-78 = Murine gammaherpesvirus 78

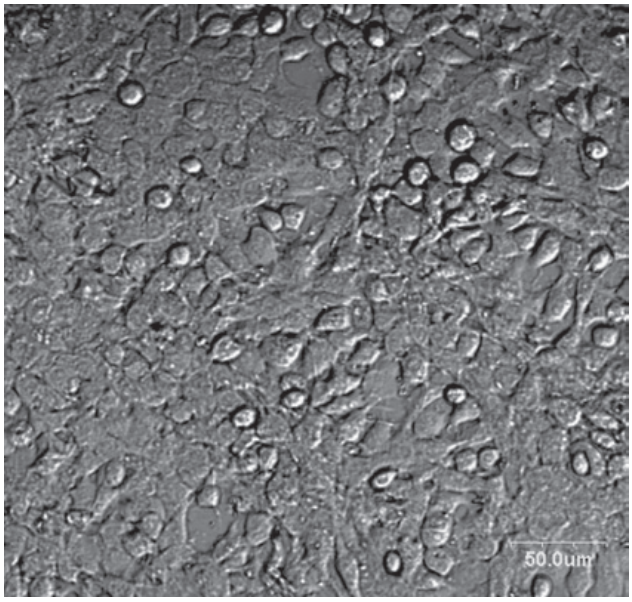

Fig. 1

Morphology of NB-78 cells

The passage 14, native preparation. Magnification 1000x.

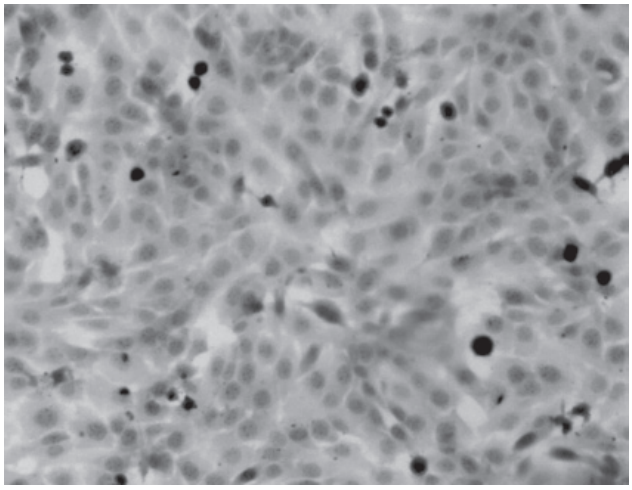

Fig. 2

Morphology of NB-78 cells

The passage 90, hematoxylin-eosine staining. Magnification 360x.

Cell lines derived from EBV-infected cells (Namalwa, Raji, Jijoye, and P3HR1) proved extremely useful in the study of virus latency (EBNA1, EBNA2, EBNA3A, EBNA3C, LMP1, LMP2- latent EBV genes) and the discovery of virus genes responsible for cell transformation (Bernasconi *et al.*, 2006).

This study was aimed at the derivation and characterization of a cell line from a lymphoma from a mouse infected with MHV-78. The morphology, karyotype and presence of viral antigen and several viral genes in the cell line were examined.

Material and Methods

Mice. Female BALB/c mice aged 4–6 weeks were used.

Virus. MHV-78 was propagated in Vero cells.

Infection of mice was performed intranasally with 2 x 10^5 PFU (20 µl) of MHV-78 per mouse under light ether anesthesia.

NB-78 cell line. A cervical lymphoma from a mouse infected with MHV-78 was minced with scissors in RPMI-1640 culture medium containing 5% of FCS. The cells were washed three times with the above medium by centrifugation and were counted after trypan blue staining. To obtain cell clones, the cells were seeded in appropriate concentration in soft agar cultures.

Soft agar cultures in 35-mm plastic Petri dishes (Nunc) consisted of a basic layer of 2.5 ml of standard medium with 0.5% agar (Difco Bacto-Agar) overlaid with 1 ml of standard medium with 0.33% agar, in which the cells were suspended. The standard medium consisted of Dulbecco's Minimum Eagle's Medium with 10% of heat-inactivated FCS and antibiotics. The cultures were incubated at 37°C in 5% CO_2. Cell clones were transferred to liquid cultures (Payne, 1981).

Liquid cultures were made in the same type of dishes using the standard medium.

IF test for detection of viral antigen in the cells was performed using a monoclonal antibody against MHV-Šumava. Viral antigen was visualized using a goat anti-mouse IgG conjugated with rhodamine (Immunotech, Slovak Republic). The positivity of cells for viral antigen was determined by counting.

A mouse monoclonal antibody against MHV-Šumava (Matušková *et al*., 2003) was used in the IF test for detection of viral antigen.

Cellular and plasmid DNA. To prepare cellular DNA, 5 x 10^6 NB-78 cells were washed with PBS and treated with a Pronase solution (0.2 mg/ml Pronase in 20 mol/l Tris-HCl pH 7.5, 150 mol/l NaCl, 2 mol/l EDTA pH 8.0, and 0.5% SDS) for 1 hr at 37°C, extracted with phenol-chloroform (1:1) and chloroform-isoamylalcohol (24:1). DNA was precipitated with equal volume of isopropanol (1:1), pelleted, washed with 70% ethanol, dried and redissolved in 200 µl of sterile water. The preparation of cellular DNA was checked by electophoresis in 0.8% agarose gel containing ethidium bromide. A plasmid with

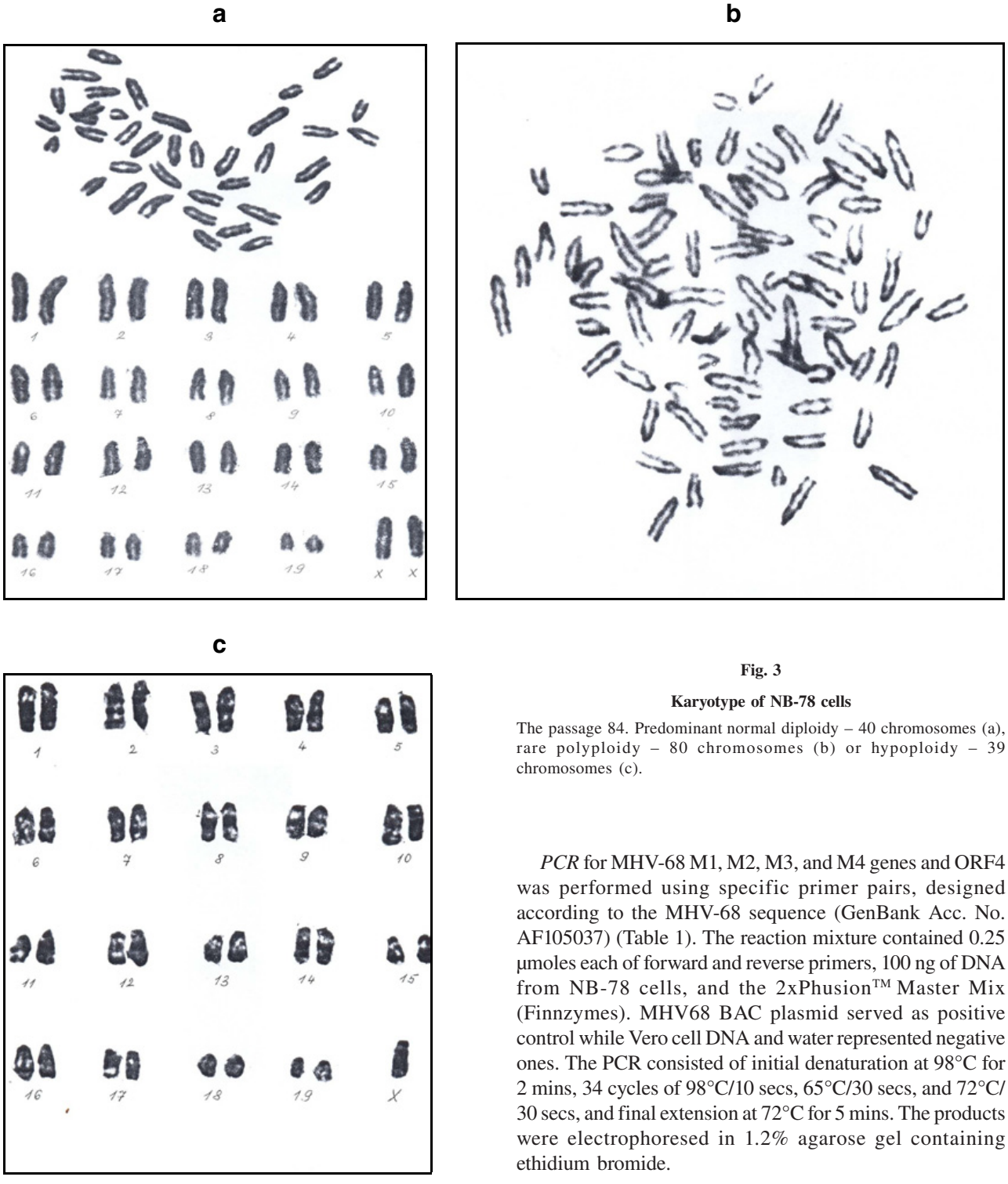

Fig. 3

Karyotype of NB-78 cells

The passage 84. Predominant normal diploidy – 40 chromosomes (a), rare polyploidy – 80 chromosomes (b) or hypoploidy – 39 chromosomes (c).

inserted MHV-68 genome (MHV68 BAC plasmid) (Adler *et al*., 2000) was produced and isolated from transformed *Escherichia coli* cultures using alkaline lysis (Maniatis *et al*., 1989).

PCR for MHV-68 M1, M2, M3, and M4 genes and ORF4 was performed using specific primer pairs, designed according to the MHV-68 sequence (GenBank Acc. No. AF105037) (Table 1). The reaction mixture contained 0.25 μmoles each of forward and reverse primers, 100 ng of DNA from NB-78 cells, and the 2xPhusion™ Master Mix (Finnzymes). MHV68 BAC plasmid served as positive control while Vero cell DNA and water represented negative ones. The PCR consisted of initial denaturation at 98°C for 2 mins, 34 cycles of 98°C/10 secs, 65°C/30 secs, and 72°C/30 secs, and final extension at 72°C for 5 mins. The products were electrophoresed in 1.2% agarose gel containing ethidium bromide.

Results and Discussion

A cell line, designated NB-78, was derived from a lymphoma which developed in cervical lymph nodes of

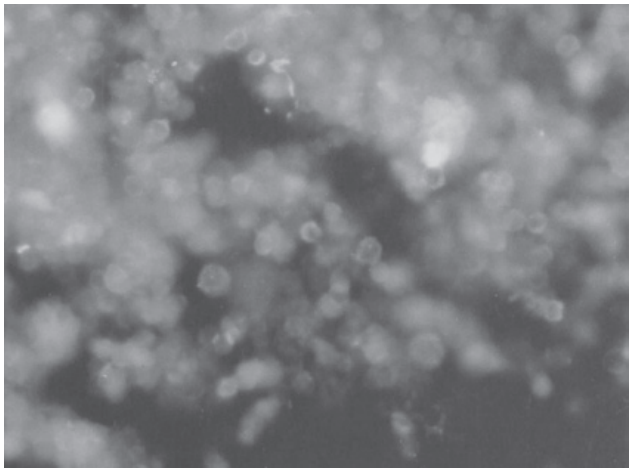

Fig. 4

Detection of viral antigen in NB-78 cells by IF test

The passage 43. Magnification 360x.

a MHV-78-infected BALB/c mouse (Mrmusová-Šupolíková *et al*., 2004). On day 252 post infection., the lymphoma was excized and trypsinized, and the cells were seeded in a soft agar culture. The obtained cell colonies were subcultured in a liquid culture. The morphology of adherent cells was initially fibroblastoid, but later it changed to a transformed epitheloid type. The cells grew to a monolayer of saturation density of 5.66 x 10^5 cells/cm^2 and doubling time of approximately 41 hrs (Figs. 1 and 2). The transformed phenotype of these cells was confirmed by their ability to produce clones in soft agar cultures. The cells displayed predominantly a normal diploid character (40 chromosomes), rarely polyploid (80 chromosomes) or hypoploid (39 chromosomes) one (Fig. 3). The cytogenetical analysis confirmed murine origin of the cell line. Its karyotype was identical with that of *Mus musculus domesticus*: 40 acrocentric chromosomes (19 autosomal pairs and XY sex chromosomes) (Hanfe *et al*., 2000).

Table 1. The primers used in this study

Amplicon size (bp)	Primer/gene	Sequence (5'-3')
674	M1(F)	GGATATGAGGGACTACTTGTTTCTGG
	M1(R)	GTCTTGGGATGATATGGGTAGGATTC
416	M2(F)	TGGCTGGATATAGACTGGTTCACTG
	M2(R)	GAGGTTTCGTTTTCAGGTAATGGC
340	M3(F)	GTCTCTTCTGGGGTGTTTGATTCTG
	M3(R)	CCTGCTCTGTCTACCCACTCTTCT
176	M4(F)	CCTCGGCATGGGATAACTATACTTCTG
	M4(R)	AATCTCTGGTACTGGCGAACTTCTGT
410	ORF4(F)	CAAATGCCCACACTAACATCTCCTAAA
	ORF4(R)	AGTATATAGGCTGGGGACTTGACTTCG

F = forward, R = reverse.

Reactivation of original virus (MHV-78) was tested at each passage in cultivation medium. However, it was proved only at the passages 43–45. The positivity of these particular passages as well as the way of directed virus reactivation are unknown. The cloning of cells with reactivated virus was not attempted.

The presence of viral antigen in the cells was examined by the IF test only occasionally. In average, viral antigen was detected in 1–2% of the cells (Fig. 4).

The presence of viral M1, M2, M3 and M4 genes and ORF4 sequence in the cells was examined only at the passage 98 and gave a positive result (Fig. 5). DNAs from uninfected Vero cells and MHV68 BAC plasmid gave

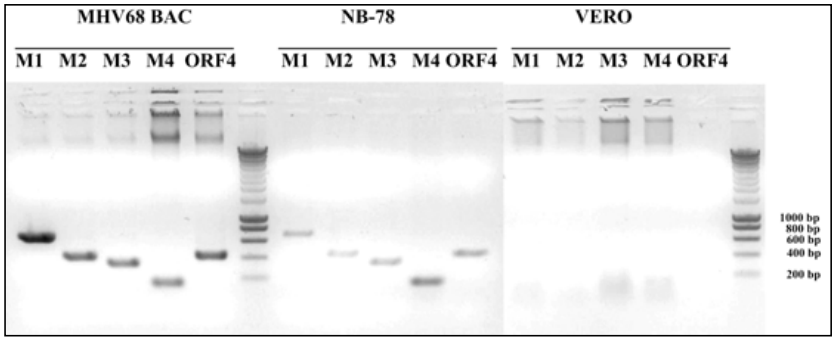

Fig. 5

Detection of MHV-68 genomic sequences in NB-78 cells by PCR

The passage 98. Agarose gel electrophoresis. The bands of 674 bp, 416 bp, 410 bp, 340 bp, and 176 bp correspond to M1, M2, ORF4, M3, and M4 genes, respectively. NB-78 cells, MHV68 BAC plasmid (positive control) and Vero cells (negative control).

expected negative and positive results, respectively. In a previous study, the M2 gene of another murine gammaherpesvirus, MHV-68, was found to be associated with latency (Husain *et al*., 1999).

In this report, we describe the first cell line derived from a MHV-78-infected animal host. This cell line, which originates from a tumor-bearing BALB/c mouse, represents a latent gammaherpesvirus infection with current absence of infectious virus, continuous but rare occurrence of viral antigen, rare reactivation of virus, transformed host cell phenotype, and the presence of virus gene sequences.

As already stated, the cell lines derived from EBV proved extremely useful in the study of virus latency and the discovery of virus genes responsible for cell transformation (Bernasconi *et al*., 2006; Sisk *et al*., 2003). Earlier, we made an unsuccessful attempt to establish a cell line from a similar virus-host system, Balb/c mice infected with MHV-72. The latter virus, similarly to MHV-78, was isolated from *Apodemus flavicollis* mice (Blaškovič *et al*., 1980). This cell line, after 60 passages, was lost apparently due to cytopathogenicity of reactivated virus (J. Mistríková, unpublished data). Four cell lines from different tumors from BALB/c mice infected with MHV-68 have been derived by Usherwood *et al.* (1996). However, only one of them harbored the respective virus genome. This may indicate a role for the virus in initiation but not continuous growth of a tumor. The virus involved in the abovementioned cell line, MHV-68, originated from the murine species *Clethrionomys glareolus* in contrast to MHV-78 and MHV-72, which were isolated from another murine species, *Apodemus flavicollis* (Blaškovič *et al*., 1980). We believe that the NB-78 cell line will become useful tool for studying the latency of gammaherpesviruses.

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