PATHOGENETIC CHARACTERIZATION OF A MOUSE HERPESVIRUS ISOLATE ŠUMAVA

J. MISTRÍKOVÁ*, T. MOŠKO, M. MRMUSOVÁ

Department of Microbiology and Virology, Faculty of Natural Sciences, Comenius University, Mlynská dolina B2, 842 15 Bratislava, Slovak Republic

Received June 28, 2001; accepted April 29, 2002

Summary. – BALB/c mice inoculated intranasally (i.n.) with the mouse herpesvirus isolate Šumava (MHV-Šumava) did not show apparent symptoms of illness. However, they showed an increase in the number of leukocytes and appearance of atypical leukocytes in peripheral blood. The infiltration of spleen with atypical cells resulted in splenomegaly. In the course of the infection the virus persisted in lungs, spleen, thymus, bone marrow, mammary glands, peritoneal macrophages and liver. We regard MHV-Šumava as one of the eight isolates of MHV-68 (a virus and species *Murid herpesvirus 4*, genus *Rhadinovirus,* subfamily *Gammaherpesvirinae,* family *Herpesviridae* (van Regenmortel *et al.*, 2000)) so far obtained. MHV-Šumava differs from MHV-68, MHV-72 and MHV-76 in some virological and pathogenetical features.

Key words: mouse herpesvirus; isolate Šumava; pathogenesis, lymphatic system

Introduction

Mouse herpesvirus isolate Šumava (MHV-Šumava) is oncogenic gammaherpesvirus which infects wild rodents and can readily infect experimental mice. MHV-Šumava is an isolate of strain MHV-68 and is closely related to both Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) and Epstein-Barr virus (EBV). This virus has been earlier shown to be oncogenic for mice (Mistríková *et al.*, 2000). MHV-Šumava has been isolated from the lungs of *Apodemus flavicollis* showing serum antibodies to MHV-68 (Mistríková and Blaškovič, 1985). MHV-Šumava has been found antigenicaly undistinguishable from other so far known isolates of MHV, namely MHV-60, MHV-68, MHV-72, MHV-76, MHV-78, MHV-Šumava, (Svobodová, *et al.*, 1982) MHV-4556, and MHV-5682 (Kožuch *et al.*, 1993). All the isolates have been found to belong to herpesviruses (Blaškovič *et al.*, 1980), but only the MHV-68 isolate has been recognized as a new virus (Mouse herpesvirus strain 68, MHV-68) and species (*Murid herpesvirus 4,* genus *Rhadinovirus,* subfamily *Gammaherpesvirinae*, family *Herpesviridae*) on the basis of the analysis of the viral genome (Efstathiou *et al.*, 1990a,b). To date we regard MHV-60, MHV-72, MHV-76, MHV-78, MHV-Šumava, MHV-4556, and MHV-5682 as different isolates of MHV-68. MHV-68 has been used extensively in the study of the virus-host interaction, especially the role of the immune system in determining the course of infection and the regulation of latency (Nash *et al.*, 1996).

First data concerning MHV-Šumava genome have been reported by Blaškovič *et al.* (1988).

The chronologically sixth MHV-Šumava isolate was originally acquired from a yellow-necked mouse *Apodemus flavicollis* using rabbit embryo fibroblasts (Mistríková and Blaškovič, 1985). The other MHV isolates have been obtained from two species of small free-living rodents via intracerebral (i.c.) passage in newborn mice (Blaškovič *at*

*E-mail: virumis@savba.sk; fax: +4212-54774284.

Abbreviations: CPE = cytopathic effect; EBV = Epstein-Barr virus; HHV-8 = *Human herpesvirus 8*; i.c. = intracerebral; IF = immunofluorescence; i.n. = intranasally; KSHV = Kaposi's sarcoma-associated herpesvirus; MHV = mouse herpesvirus; MHV-Šumava = mouse herpesvirus isolate Šumava; MAb = monoclonal antibody; p.i. = post infection

al., 1980). MHV-60, MHV-68 and MHV-72 were isolated from *Clethrionomys glareolus,* while MHV 76 and MHV-78 from *Apodemus flavicollis.*

According to physical and chemical properties MHV-Šumava seemed to be the most different from the other MHV isolates (Blaškovič *et al.*, 1988). The aim of this study was characterization of some virological and pathological features of MHV-Šumava infection in BALB/c mice and assessment of similarities and differences between MHV-Šumava and the other MHV isolates.

Materials and Methods

Mice. Female Balb/c mice were obtained from the Institute of Virology, Slovak Academy of Sciences, Bratislava and infected at 6 weeks of age.

Virus. A MHV-Šumava stock was prepared by infection of Vero cells and harvesting at 72 or 96 hrs post infection (p.i.). Cells were disrupted and the virus was released by homogenization and sonication. The obtained viral suspension was clarified by low-speed centrifugation. The supernatant was stored at -70°C until used. Infectious virus was assayed by plaque titration in Vero cells (see below).

Experiments with mice. Seventy female 4–6 week-old Balb/c mice were inoculated intranasally (i.n.) with 2 x 10^5 PFU (20 µl) of the virus per mouse under light anaesthesia with ether. Newborn 2–3-day old mice were inoculated perorally with 1 x 10^5 PFU (10 µl) of ml of the virus per mouse. Mice were killed at different times p.i. by cervical dislocation. The blood, lungs, spleen, thymus, bone marrow, peritoneal macrophages, liver, and kidneys were removed and used for preparation of cell suspensions for detection of viral antigen by immunofluorescent test. Specimens for detection of the virus were taken and stored at -70°C until virus titration.

Blood samples were taken from *sinus orbitalis* at different times p.i. for serum preparation and examination of leukocytes. Blood samples were mixed with heparin (final concentration of 2–4 U/ml) to prevent blood clotting. The obtained sera were inactivated at 56°C for 30 mins and stored at -20°C until titrated.

Staining of leukocytes. Blood smears were made immediately after blood collection. They were stained after fixation by air drying with the May-Grünwald solution for 10 mins and the Giemsa Romanowski solution for 15 mins. Number of leukocytes was determined after 10 mins of staining with the Türk solution.

Differential white blood cell count. Blood picture consisted of calculation of percentage of each kind of white blood cells.

Titration of infectious virus was done in Vero cells. Frozen homogenates of organs were thawed and clarified by a low-speed centrifugation and then diluted 10-fold serially up to 10^{-6} in Dulbecco's Modified Eagle's Medium supplemented with 3% of inactivated bovine serum, glutamine (300 mg/l) and gentamicine (80 mg/l). Each dilution (0.1 ml per culture vessel) was used for infecting Vero cell monolayers. After 2–7 days of incubation at 37°C in 5% CO_2 the monolayers were fixed and stained and the cytopathic effect (CPE) was evaluated.

Cytological examination. Infected cell cultures grown on glass cover slips were fixed in Carnoy solution for 15 mins and then transferred to 96% ethanol. The fixed specimens were stained with hematoxylin-eosine.

Immunofluorescence (IF) test. The presence of the virus antigen in cells from organs of infected mice was determined by indirect IF test and the percentage of positive cells was calculated. Suspensions of cells (100 µl) obtained from lymphatic tissues of infected mice were stained with a monoclonal antibody (MAb) prepared against MHV-Šumava (MAb/MHV-Šumava) diluted 1:100–1:500 and with a goat anti-mouse IgG (H+L) conjugated with rhodamine (Immunotech, Slovak Republic).

Virus neutralization test was performed with 2-fold dilutions of sera from infected mice to which 100–1000 ID_{50} of appropriate virus was added. The virus-serum mixtures were incubated at 37°C for 90 mins and then inoculated into 24–48-hrs-old Vero cell cultures according to the growth requirements of the respective virus. The titer was calculated after 5–6 days when a complete CPE was observed in control.

Results and Discussion

Lethality of MHV-Šumava for mice

MHV-Šumava was lethal for newborn suckling mice which died 15–16 days after peroral infection in contrast to 4–6-week-old mice which did not show apparent symptoms of illness while 48% of 6-week-old mice infected with MHV-68 developed apparent symptoms of illness at that time (Sunil-Chandra *et al.*, 1992a).

Persistent MHV-Šumava infection in mice

The presence of the virus was followed in the lymphatic system of seventy 4–6-week-old female Balb/c mice infected intranasally with 2 x 10^5 PFU of MHV-Šumava per mouse. The mice were sacrificed on days 2–180 p.i. and samples of blood, lungs, spleen, thymus, bone marrow, mammary glands, peritoneal macrophages, liver and kidneys were taken (Table 1). Peritoneal macrophages were obtained by perfusion. Days 2, 3, 5, 7, 10, 14, 28, 90 and 180 p.i. were chosen for sacrification on the basis of our previous experience with MHV-72 (Mistríková *et al.*, 1994). We detected the highest infectious titer of MHV-Šumava during the acute infection between days 7 and 14 in lungs. Intranasal inoculation with MHV-68 leads to an initial lung infection, but in comparison with MHV-Šumava one week earlier with peak titers of virus in lungs 5–7 days p.i. (Sunil-Chandra *et al.*, 1992a). Furthermore, we observed the highest titer of MHV-Šumava between days 28 and 90 p.i. in splenocytes and between days 10 and 28 p.i. in peritoneal macrophages; the latter increase was accompanied by an increase in virus neutralization antibodies. The persistence of the virus in spleen, thymus and peritoneal macrophages for a long time confirmed the affinity of MHV-Šumava to lymphatic system.

Table 1. Detection of infectious virus, viral antigen and virus-neutralizing antibodies in 4–6-week-old Balb/c mice infected with MHV-Šumava

Organ		Day p.i. 2	3	5	7	10	14	28	90	180
Lungs	$TCID_{50}$/ml	10^1	10^1	10^1	10^2	10^3	10^2	1	0	0
	IF	+	+	+	+++	+++	+++	++	+	+
Spleen	$TCID_{50}$/ml	0	1	0	0	10^1	10^1	10^2	10^3	10^2
	IF	–	–	–	+	++	+	+++	++	+
Thymus	$TCID_{50}$/ml	0	1	0	0	1	10^1	10^1	10^1	10^2
	IF	–	–	–	–	–	+	+	+	+
Bone marrow	$TCID_{50}$/ml	0	1	0	0	10^1	0	0	0	0
	IF	–	–	+	–	+	+	++	+	+
Peritoneal macrophages	$TCID_{50}$/ml	0	0	0	1	10^2	10^2	10^2	0	10^1
	IF	–	–	–	–	+	+	++	–	–
Mammary glands	$TCID_{50}$/ml	0	10^1	0	0	10^1	10^1	0	0	10^1
	IF	–	+	–	–	+	+	–	–	+
Liver	$TCID_{50}$/ml	0	0	0	0	1	1	0	0	0
	IF	–	–	–	–	–	–	–	–	–
Kidneys	$TCID_{50}$/ml	0	0	0	0	0	0	0	0	0
	IF	–	–	–	–	–	–	–	–	–
Titer of Ab		ND	0	0	64	128	512	256	64	32

Mice were infected i.n. with 2 x 10^5 PFU of MHV-Šumava per mouse. IF = immunofluorescence evaluated as (–) for all cells negative, as (+) for 2–4% of cells positive, as (++) for 4–10% of cells positive, and as (+++) for 50% of cells positive. Titer of antibody (Ab) expressed as inverted value of the highest positive dilution of Ab. 0 = no virus detected either in the 1st or in the 2nd cell culture subpassage. ND = not done.

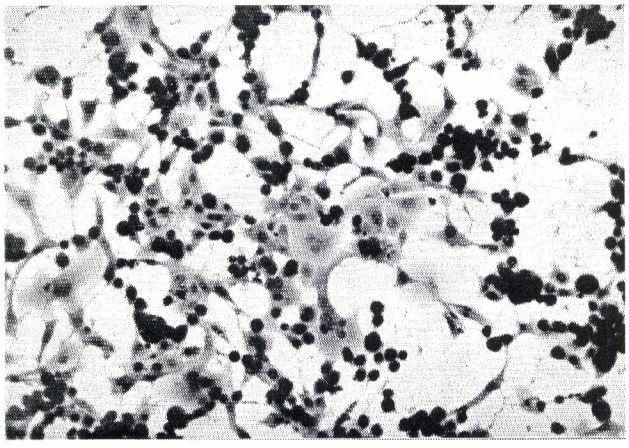


Fig. 1
Vero cells at 72 hrs p.i. with a suspension from lungs of MHV-Šumava infected Balb/c mice

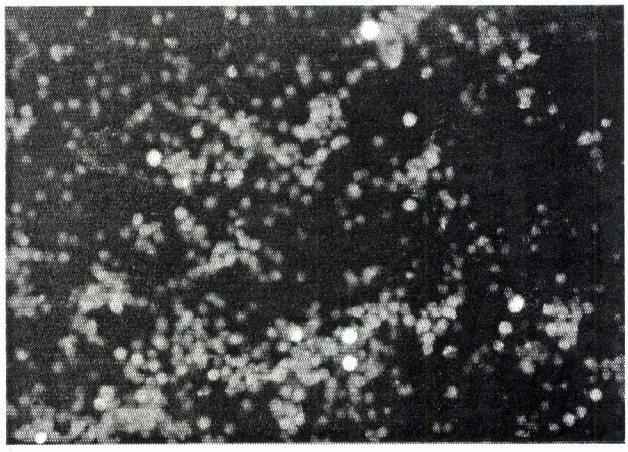


Fig. 2
IF of MHV-Šumava antigen in splenocytes from Balb/c mice at day 14 p.i.

Magnification 360x.

The presence of the virus in the lungs after i. n. inoculation was examined indirectly by infecting Vero cells with suspension obtained from lungs of MHV-Šumava infected mice 72 hrs p.i. (Fig. 1). The presence of the virus antigen in spleen and peritoneal macrophages was demonstrated by IF test on Fig. 2 (splenocytes) and Fig. 3 (peritoneal macrophages).

Effect of MHV-Šumava infection on mouse leukocytes

We also studied the effect of MHV-Šumava infection on the number and morphology of leukocytes in peripheral blood of Balb/c mice. The number of leukocytes increased during the acute infection, reaching a maximum on day 28 p.i. (Figs. 5a,b). Atypical leukocytes (blastic forms of

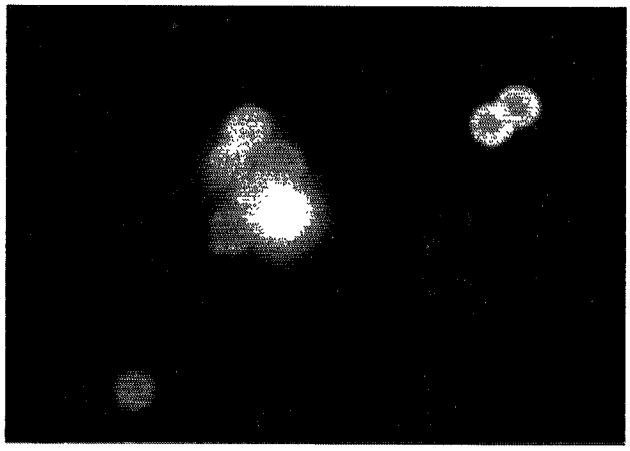


Fig. 3
IF of MHV-Šumava antigen in peritoneal macrophages from Balb/c mice at day 28 p.i.

Magnification 720x.

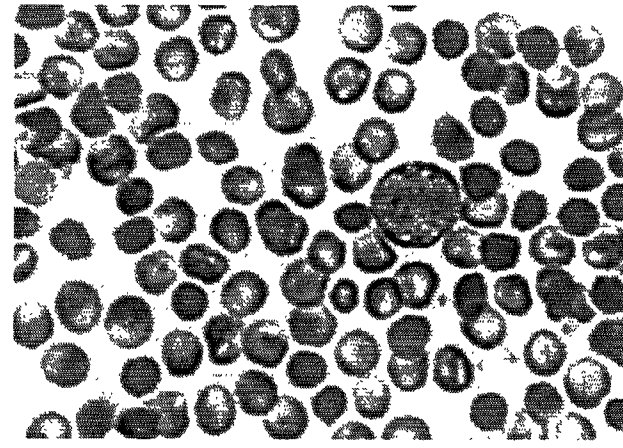
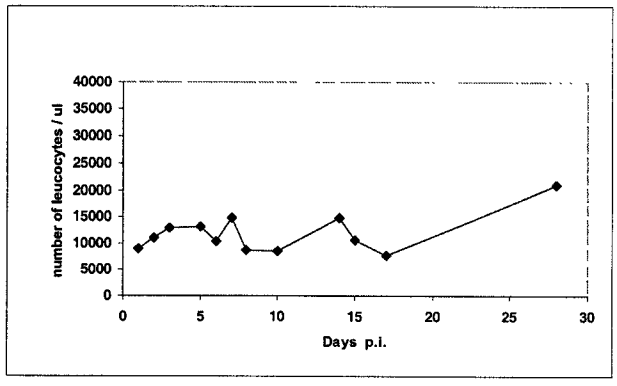


Fig. 4
Atypical leukocytes (blastic forms of leukocytes) in peripheral blood of Balb/c mice infected with MHV-Šumava

Magnification 1000x.



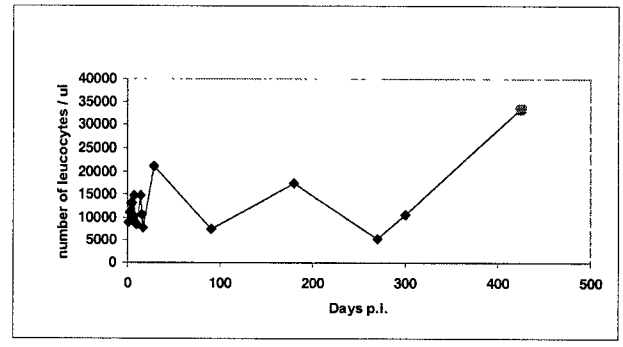


Fig. 5a,b
Effect of MHV-Šumava infection on the number of leukocytes in peripheral blood of Balb/c mice

leukocytes) were observed in differential white blood cell count. The number of atypical leukocytes increased during the acute infection, reaching a maximum on days 5 and 10 p.i. (Figs. 6a,b).

Further we investigated the number of splenocytes for each spleen. We found that the number of splenocytes increased in the course of acute infection with a maximum at days 6–10 p.i. (Fig. 7a). Nash *et al.* (1996) have reported that with MHV-68 there was a marked transient splenomegaly at days 12–14 p.i. with a double total splenocyte number. In the present study, we detected a significant increase in the number of splenocytes in the course of chronic infection with MHV-Šumava, namely comparing days 400 and 0 p.i. (Fig. 7b).

Interestingly, the MHV-Šumava infection of newborn mice resulted in infection of neural cells. We observed developing encephalitis in 4 of 10 newborn suckling mice after peroral administration of the virus.

After peroral infection of mice with MHV-68 viremia leads to a detectable virus in heart, kidneys, adrenal glands and major lymphoid organs but not in neural tissues during 10–15 days p.i. (Rajčáni *et al.*, 1985). We detected viral antigen in brain cells from one mouse developing tumor and isolated the virus from brains of adult mice infected with MHV-Šumava. It is the first reported isolation of the virus from brain of adult mice infected with MHV-Šumava.

We detected the virus in mammary glands on days 3, 9, 14 and 180 p. i.; this finding also indicates the possibility of secretion of the virus by maternal milk similarly to EBV.

A long-term infection with MHV-Šumava has led to the development of a lymphoproliferative disease similarly to

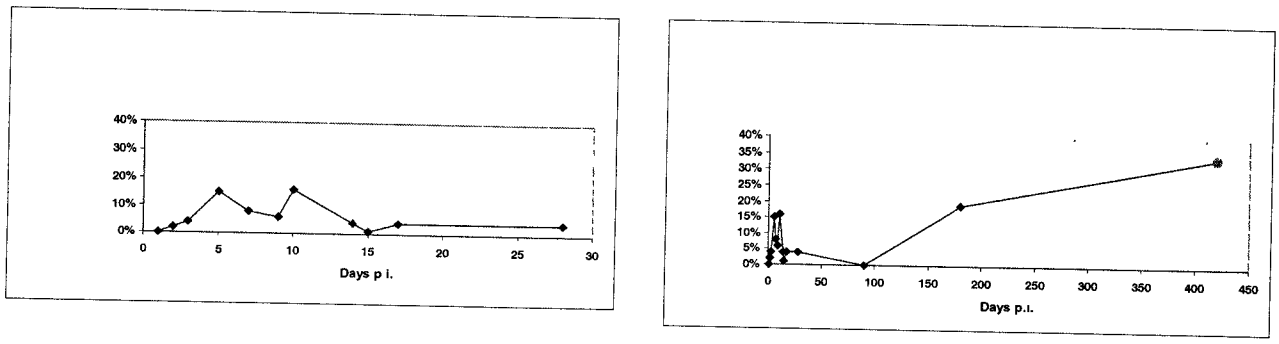


Fig. 6a,6b
Effect of MHV-Šumava infection on the percentage of atypical leukocytes in peripheral blood of Balb/c mice

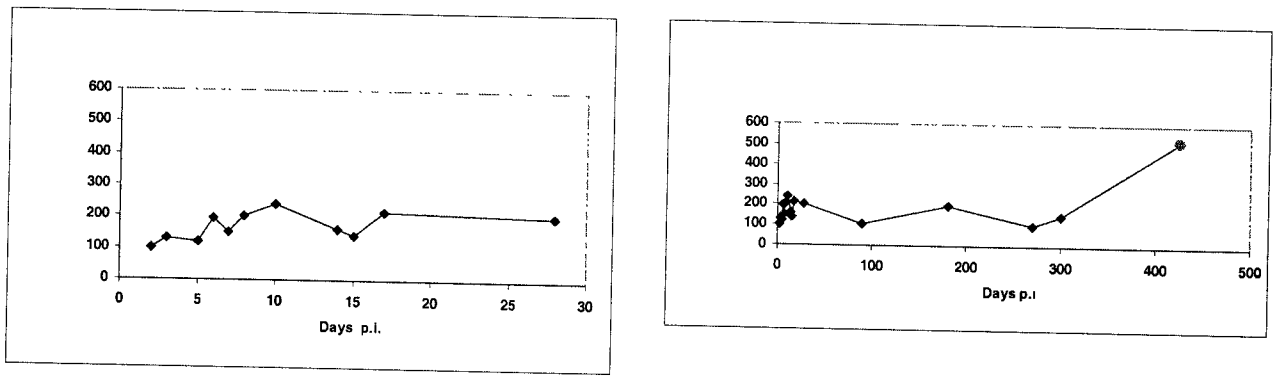


Fig. 7a,7b
Effect of MHV-Šumava infection on the number of splenocytes in peripheral blood of Balb/c mice

MHV-68 (Sunil-Chandra *et al.*, 1994) and MHV-72 (Mistríková *et al.*, 1996; Mistríková and Mrmusová 1998).

Mistríková *et al.* (2000) have shown on 100 BALB/c mice infected with MHV-Šumava that, during 2 years, 14.6% of them developed tumors as compared to 3% in non-infected mice.

The most infected organs in MHV-Šumava infection were lungs, spleen and peritoneal macrophages. Here it should be mentioned that MHV-Šumava has been isolated from lungs of a seropositive wild mouse. The MHV-68 persistence in the lungs at low levels has been demonstrated (Blaškovič *et al.*, 1984; Rajčáni *et al.*, 1985; Usherwood *et al.*, 1996). In detecting MHV-Šumava in the lungs and spleen we succeeded by using an indirect assay, which involved co-cultivation of infected cells with Vero cells and an indirect IF test.

Infection of the spleen was significantly delayed in MHV-Šumava-infected BALB/c mice at days 28–90 p.i. in comparison to MHV-72 at days 7–28 p.i. (Mistríková *et al.*, 1994), MHV-68 at day 14 p.i.) and MHV-76 (at days 6–10 p.i.) (Macrae *et al.*, 2001). MHV-Šumava developed splenic latency and splenomegaly in contrast to MHV-76 with which no splenomegaly has been observed (Macrae *et al.*, 2001).

Strain MHV-68 has been shown to be a B-lymphotropic virus (Sunil-Chandra *et al.*, 1992b). In the present study MHV-Šumava infected not only B lymphocytes but also peritoneal macrophages and thymocytes similarly to MHV-72 (Mistríková *et al.*, 1994).

Oncogenicity of MHV-Šumava

MHV-Šumava has been earlier shown to be oncogenic (Mistríková *et al.*, 2000). In the present study we detected 14.6 % of tumors in BALB/c mice similarly to earlier data, namely 13% for MHV-72 (Mistríková *et al.*, 2000) and 11% for MHV-68 (Sunil-Chandra *et al.*, 1994).

Here it should be mentioned that MHV-Šumava was isolated in another locality and five years later as compared to other MHV isolates. Furthermore, MHV-Šumava similarly

to MHV-76 did not cause cytomegalia formation, the phenomenon not usually observed in Vero cells anyway.

Conclusions

MHV-Šumava is characteristic by (i) a lytic infection of Vero cells and persistence in lymphoid or possibly macrophage cells, (ii) a more efficient replication in tissue cultures in comparison to MHV-68, its virus titer on rabbit embryo fibroblasts being 10^{10} $TCID_{50}$/ml as compared to 10^{6} $TCID_{50}$/ml for MHV-68, (iii) an increase in number of leukocytes and appearance of atypical leukocytes in peripheral blood of Balb/c mice, and (iv) enlargement of the spleen as a consequence of infiltration by atypical leukocytes. MHV-Šumava differs from MHV-68, MHV-72 and MHV-76 in some viological and pathogenetical features.

Acknowledgement. This work was supportet by the grant 1/61 60/ 99 of the Grant Agency of the Ministry of Education of Slovak Republic and Slovak Academy of Sciences.

Note. The autors claim that all procedures using animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientifical Purposes from 1986.

References

Blaškovič D, Stančeková M, Svobodová J, Mistríková J (1980): Isolation of five strains of herpesviruses from two species of free living small rodents. *Acta Virol.* **24**, 468.

Blaškovič D, Staneková D, Rajčáni J (1984): Experimental pathogenesis of murine herpesvirus in newborn mice. *Acta Virol.* **28**, 225–231.

Blaškovič D, Sekeyová Z, Turňa J, Kúdelová M, Slávik I, Mucha V (1988): Purification of murine alphaherpesvirus and some properties of its DNA. *Acta Virol.* **32**, 329–333.

Efstathiou S, Ho YM, Minson AC (1990a): Cloning and molecular characterization of the murine herpesvirus 68 genome. *J. Gen. Virol.* **71**, 1355–1364.

Efstathiou S, HoYM, Hall S, Styles CJ, Scott SD, GompelsUA (1990b): Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. *J. Gen. Virol.* **71**, 1365–1372.

Kožuch O, Reichel M, Leššo J, Remeňová A, Labuda M, Lysý J, Mistríková J (1993): Further isolation of murine herpesviruses from small mammals in southwestern Slovakia. *Acta Virol.* **37**, 101–105.

Macrae IA, Dutia BM, Milligan S, Browstein DG, Allen DJ, Mistríková J, Davison AJ, Nash AA, Stewart JP (2001): Analysis of a novel strain of murine gammaherpesvirus reveals a genomic locus important for acute pathogenesis. *J. Virol.* **75**, 5315–5327.

Mistríková J, Blaškovič D (1985): Ecology of the murine alphaherpesvirus and its isolation from lung of rodents in cell culture. *Acta Virol.* **29**, 312–317.

Mistríková J, Remeňová A, Leššo J, Stančeková M (1994): Replication and persistence of murine herpesvirus 72 in lymphatic system and peripheral blood mononuclear cells of BALB/c mice. *Acta Virol.* **38**, 151–156.

Mistríková J, Rajčáni J, Mrmusová M, Oravcová I (1996): Chronic infection of BALB/c mice with murine herpesvirus 72 is associated with neoplasm development. *Acta Virol.* **40**, 297–301.

Mistríková J, Mrmusová M (1998): Detection of abnormal lymphocytes in the blood of BALB/c mice infected with murine gammaherpesvirus strain 72: the analogy with Epstein-Barr virus infection. *Acta Virol.* **42**, 79–82.

Mistríková J, Rašlová H, Mrmusová M, Kúdelová M (2000): A murine gammaherpesvirus. *Acta Virol.* **44**, 211–226.

Nash AA, Usherwood EJ, Stewart JP (1996): Immunological features of murine gammaherpesvirus infection. *Semin. Virol.* **7**, 125–130.

Rajčáni J, Blaškovič D, Svobodová J, Čiampor F, Hučková D, Staneková D (1985): Pathogenesis of acute and persistent murine herpesvirus infection in mice. *Acta Virol.* **29**, 51–60.

Svobodová J, Stančeková M, Blaškovič D, Mistríková J, Leššo J, Russ G, Masárová P (1982): Antigenic relatedness of alphaherpesviruses isolatedfrom free living rodents. *Acta Virol.* **26**, 438–443.

Sunil-Chandra NP, Efstathiou S, Arno J, Nash AA (1992a): Virological and pathological features of mice infected with murine gammaherpesvirus 68. *J. Gen. Virol.* **73**, 2347–2356.

Sunil-Chandra NP, Efstathiou S, Nash AA (1992b): Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes *in vivo*. *J. Gen. Virol.* **73**, 3275–3279.

Sunil-Chandra NP, Arno J, Fazakerley J, Nash AA (1994): Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. *Am. J. Pathol.* **145**, 818–826.

Usherwood EJ, Stewart JP, Robertson K, Allen DJ, Nash AA (1996): Absence of splenic latency in murine gammaherpesvirus 68-infected B cell-deficient mice. *J. Gen. Virol.* **77**, 2819–2825.

van Regenmortel MHV, Fauquet CM, Bishop DHL (2000): *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*. Academic Press, San Diego, San Francisco-New York-Boston-London-Sydney-Tokyo.