Pathogenetical Characterization of MHV-76: a Spontaneous 9.5-Kilobase-Deletion Mutant of Murine Lymphotropic Gammaherpesvirus 68

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Abstract

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Murid gammaherpesvirus 4 (MuHV-4) provides a small animal model for the study of animal gammaherpesviruses. MHV-76 is a spontaneous deletion mutant as compared to the prototype strain of MuHV-4 (MHV-68). The MHV-76 genome lacks at least 12 ORFs at the 5´-end including the M1, M2, M3 and M4 genes and the eight viral t-RNA-like genes. During 27 months of experimental infection of BALB/c mice we followed their pathogenesis, immunology and oncogenic properties. After intranasal infection with MHV-76, the infectious virus was detected in the blood, thymus, lungs, heart, liver, spleen, bone marrow, peritoneal macrophages, lymph nodes, kidneys, mammary glands, brain and small intestine. The acute phase of infection was attenuated, but the chronic phase of infection was accompanied with long persistence of virus not only in the lymphatic, but in the neural and glandular tissue, as well. In comparison with the prototype strain, splenomegaly and lymphocytosis was very low. Surprisingly, during 27 months the BALB/c mice infected with MHV-76 did not develop lymphoproliferative disorders like infectious mononucleosis, leukaemia or lymphomas. We hypothesize that the M4 gene, present in all oncogenic MHV isolates, might be related (directly or indirectly) to their transforming properties.

Mouse herpesvirus 76, deletion mutant, pathogenesis, oncogenesis, M4 gene, acute infection, chronic infection, atypical lymphocytes, lymphoproliferation

The interest in biology of gammaherpesviruses stems largely from their association with a variety of malignancies. The Human Epstein-Barr virus (EBV) and animal Herpesvirus Saimiri (HVS) are associated with lymphoproliferative disorders. MHV-68 along with isolates 60, 72, 76, 78 and Šumava belongs to the MuHV-4 species, a member of Rhadinovirus genus of the subfamily Gammaherpesvirinae (Fauquet et al. 2005).

MHV-68 represents a small animal model for the study of gammaherpesvirus pathogenesis. MHV-68 established a latent infection in B-lymphocytes, following an acute respiratory infection. In late stages of a long-term infection, 10% of mice developed a lymphoproliferative disease. A high proportion of these mice (50%) displayed high-grade lymphomas (Sunil-Chandra et al. 1994; Mistríková et al. 1996). MHV-60, MHV-68, MHV-72, MHV-78 and MHV-Šumava have an oncogenic potential including tumours in 22%, 9%, 11%, 7% and 14.5%, respectively (Pappová et al. 2004; Sunil-Chandra et al. 1994; Mistríková et al. 1996, 2002; Mrmusová-Šupolíková et al. 2003). Our research was focused on MHV-76, another isolate of MHV-68 (Blaškovič et al. 1980), which was isolated at the same time as MHV-68, but from a different murid species - a yellow-necked wood mouse *Apodemus flavicollis*. MHV-76 is supposed to be identical to MHV-68, except for genes M1, M2, M3 and M4 as well as eight vt-RNAs on the left end of the genome. On the basis of growth characteristics in cell culture and the cytopathic effect, MHV-76 was originally described as an alphaherpesvirus (Svobodová et al. 1982), Reichel et al. (1991)

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found resemblance in the polypeptide profiles of MHV-76 and Herpes simplex in the rabbit embryo fibroblast cell, but it could be accidental due to glycolysation and phosphorylation of particular polypeptides. However, genomic analysis of MHV-68 (Efstathiou et al. 1990) classified it as gammaherpesvirus. Hamelin and Lussier (1992) used restriction endonuclease, dot-blot hybridization and cross-hybridization to show a high level of DNA homology between MHV-76 and rat cytomegalovirus (a betaherpesvirus). They hypothesized MHV-76 is a possible member of all 3 subfamilies. Macrae et al. (2001) on the basis of molecular analysis of MHV-76 concluded that MHV-76 has a genome structure nearly identical to that of MHV-68, except a 9.5 kbp deletion that contains the M1, M2, M3 and M4 and eight viral t-RNA-like genes. On the basis of MHV-76 analysis authors reported that this locus is important for acute pathogenesis, including acute replication and establishment of MHV-68 latency in the spleen following intranasal infection. Since MHV-76 is supposed to be identical to oncogenic MHV-68, except for deletion genes, we wanted to analyze how these deletions influence the pathogenesis during long-term infection and the oncogenic potential.

Materials and Methods

Virus

The MHV-76 stock was prepared by infection of VERO cells and harvesting at 72 or 96 h post infection (p.i.). Cells were disrupted and the virus was released by homogenization and sonication. The supernatant was stored at -70 °C until use. Infectious virus was assayed by plaque titration in VERO cells.

Animals

BALB/c mice coming from the Faculty of Veterinary Medicine, Brno, Czech Republic were kept at the Institute of Virology, Slovak Academy of Sciences, Bratislava, under standard housing conditions. Eighty of 100 female 4 - 6-week-old mice were inoculated intranasally (i.n.) with 2.10^5 PFU (20 µl) of the virus per mouse under light anaesthesia with ether. Twenty of 100 non-infected 4 - 6-week-old mice served as controls. All procedures using animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes from 1986.

Experiments with mice

Mice were killed at different times during acute infection (3, 5, 7, 12, 14, 20, 25, 28 d.p.i.) and chronic infection (1, 3, 5, 7, 9, 11, 13, 15, 17, 24, 27 months p.i.) by cervical dislocation. The blood, thymus, lungs, heart, liver, spleen, bone marrow, peritoneal macrophages, lymph nodes, kidneys, mammary glands, brain, and small intestine were removed and used for preparation of cell suspensions for detection of virus and viral antigen by immunofluorescence test. Specimens for detection of the virus were collected and stored at -70 °C until virus titration. Blood samples were taken from sinus orbitalis at different times during acute infection (3, 5, 7, 12, 14, 20, 25, 28 d.p.i.) and chronic infection (1, 3, 5, 7, 9, 11, 13, 15, 17, 24, 27 months p.i.) instantly with heparin (final concentration of 2 - 4 U/ml to prevent blood clotting).

Staining of blood elements

Blood smears were made immediately after blood collection. After fixation by air drying they were stained by May-Grünwald solution for 10 min and then by Giemsa-Romanowski solution for 15 min. Stain solution was removed by rinsing with tap water and smears were examined by microscopy with immersion oil. Differential white blood cell count was done by calculation of percentage of each kind of white blood cells.

The amount of 25 ml of unclotted blood was added to 475 µl of Turk solution for 10 min and then the number of leukocytes was determined.

Titration of virus

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

Immunofluorescence (IF) test

The presence of virus antigen in cells from organs of infected mice was determined by indirect IF test and the percentage of positive cells was calculated. Suspension of cells (100 µl) obtained from organ tissues of infected mice was washed with PBS prior to staining with a monoclonal antibody (Mab) against MHV (dilution 1 : 100 - 1 : 500) for 45 min at 37 °C. The monoclonal antibody was prepared in our laboratory (Matušková

et al. 2003). After three washes in PBS the samples were stained with a goat anti-mouse IgG (H + L) conjugated with rhodamine (Immunotech, Slovak Republic) and incubated at 37 °C for 45 min. Followed by washing with PBS three times, the cells were mounted in mounting solution and examined under fluorescence.

Neutralisation assay

Sera obtained from infected mice were heat-inactivated (56 °C for 30 min) and diluted in Dulbecco's Modified Eagle's Medium (supplemented with 2% of heat-inactivated bovine serum, glutamine and gentamicin) with an initial serum dilution of 1 : 2. The tissue culture infectious dose ($TCID_{50}$) of the virus stocks was determined, diluted sera and virus samples were mixed (100 µl of each), and the mixture was incubated at 37 °C for 1 h. The virus-serum mixtures were then inoculated on the VERO cell monolayer in a 96-well dish and monitored for the development of the cytopathic effect. The neutralising titre of a particular serum was defined as the reciprocal of 50% of the highest dilution that resulted in no observable cytopathic effect.

Results

One hundred BALB/c mice were used for the experiment. Eighty of 100 were infected with MHV-76 and 20/100 served as non-infected control. Infected and non-infected groups of mice were observed for 27 months. MHV-76 in comparison with the prototype strain MHV-68 did not show apparent symptoms of illness. Thirteen specimens from different organs were observed for the presence of virus: blood, thymus, lungs, heart, liver, spleen, bone marrow, peritoneal macrophages, lymph nodes, kidneys, mammary glands, brain, and small intestine of 4 - 6 week-old BALB/c mice infected intranasally with 2.10^5 PFU of MHV-76 per mouse. Four of the infected mice and one non-infected mouse were sacrificed on days 3, 5, 7, 12, 14, 20, 25, 28 d.p.i., during acute infection and in month 1, 3, 5, 7, 9, 11, 13, 15, 17, 24, 27 during chronic infection. Blood samples were taken at certain intervals chosen on the basis of our previous experiments with MHV-72 (Mistríková et al. 1994), MHV-Šumava (Mistríková et al. 2002), MHV-78 (Mrmusová-Šupolíková et al. 2003) and MHV-60 (Pappová et al. 2004). Results of our experiments are demonstrated in Table 1 (acute infection) and Table 2 (chronic infection). Infectious virus and virus antigen was detected by cocultivation with permissive VERO cells and indirect immunofluorescence using monoclonal antibodies prepared against MHV. During the acute phase of infection

Table 1. Detection of infectious virus and viral antigen in 4-6-week-old BALB/c mice infected with MHV-76 during acute phase of infection

Organ	3dpi	5dpi	7dpi	12dpi	14dpi	17dpi	20dpi	25dpi	28dpi
Blood	-	+	+	-	-	+	+	+	+
Thymus	+	+	+	+	+	-	-	-	+
Lungs	+	++	±±	-	+	+	+	-	+
Heart	-	+	+	-	+	+	+	+	+
Liver	+	+	+	+	+	+	+	+	+
Spleen	-	-	+	+	+	+	-	+	+
Bone marrow	-	+	-	-	+	+	+	-	-
Peritoneal macrophages	-	-	-	-	+	+	+	+	+
Lymph nodes	-	+	+	+	+	+	+	+	+
Kidney	-	+	+	+	+	+	+	+	-
Mammary glands	ND	ND	ND	ND	ND	ND	ND	ND	ND
Brain	-	-	+	+	-	+	+	-	+
Small intestine	-	-	-	-	-	-	+	-	+

Mice were infected i.n. with 2.10^5 PFU of MHV-76 per mouse. Titre of virus evaluated as (-) negative, (+) positive specimens only in concentrated suspension, (++) positive specimens in dilution 10^{-1}. (±±) positivity of speciment confirmed with immunofluorescent method -IF , ND = not done

Table 2. Detection of infectious virus and viral antigen in 4-6-week-old BALB/c mice infected with MHV-76 during chronic phase of infection

Organ	1mpi	3mpi	5mpi	7mpi	9mpi	11mpi	13mpi	15mpi	17mpi	24mpi	27mpi
Blood	++	-	+++	-	-	++	+	-	-	-	+
Thymus	-	+	+	++	-	+	+	+	+	++	+
Lungs	+	-	++	-	++	++	+	-	-	-	-
Heart	+	-	++	+	-	++	-	-	-	++	++
Liver	-	-	+++	+++	++	±±±	++	+++	++	+++	±±±
Spleen	+		+	+++	+++	±±	++	++	++	±±±	-
Bone marrow	+	+	++	+	++	+	++	+	-	++	+
Peritoneal macrophages	+	+	+	++	+	+	+	+	-	+	-
Lymph nodes	+	+	++	+	+	+	++	++	+	+	-
Kidney	+	+	++	+	++	++	++	++	+	++	++
Mammary glands	-	+	-	+	+	++	+	+++	+	-	-
Brain	+	+	-	+++	+	+	+	++	-	-	±±
Small intestine	+	++	++	++	±±	±±	++	++	++	±±	±±

Mice were infected i.n. with 2.10^5 PFU of MHV-76 per mouse. Titre of virus evaluated as(-) negative, (+) positive specimens only in concentrated suspension, (++) positive specimens in dilution 10^{-1}, (+++) positive specimens in dilution 10^{-2}. (±±)/(±±±) positivity of speciment confirmed with immunofluorescent method -IF

the highest virus titre from all investigated organs was observed in the lungs, but in comparison with the prototype strain MHV-68, the titre of virus was very low. Also in other organs titres of virus were significantly decreased. The character of chronic infection of MHV-76 (Table 2) was obviously different from the prototype strain infection as well as other MHV isolates. Establishment of latency was attenuated and virus persisted in most organs, especially in the liver, small intestine, spleen and kidneys. Surprisingly, we also detected MHV-76 in the brain.

Table 3a. Effect of MHV-76 infection on the number of leukocytes and on the presence of atypical lymphocytes of BALB/c mice in acute infection

	3dpi	5dpi	7dpi	12dpi	14dpi	20dpi	25dpi	28dpi	Control
Number of leukocytes/ 1 µl blood	11100	11820	6300	18 900	8260	8500	1000	13600	8000
% of atypical lymphocytes	3%	9.6%	8.8%	5%	6.1%	4%	4.2%	3.5%	0%

Atypical leukocytes observed in MHV-76 acute infected mice are similar to atypical lymphocytes in EBV infected human blood in course IM.

In the second part of our study the analysis of differential white blood cell count of healthy/control and virus-infected BALB/c mice was followed (Tables 3a, 3b). The obtained results showed that the infection with MHV-76 was not accompanied with changes in the number of leukocytes and quality of differential white blood cell count in correlation with the duration of infection. During acute infection the frequency of atypical leukocytes was very low, reaching a maximum of 9.6% on day 5 p.i. In comparison with the control group, mice infected with MHV-76 had a slightly increased number of leukocytes with the highest numbers on day 12 p.i. 18 900 leu/µl blood. During the acute and chronic phases of infection we did not observe significant splenomegaly. We investigated blood serum for MHV-76

Table 3b. Effect of MHV-76 infection on the number of leukocytes and on the presence of atypical lymphocytes of BALB/c mice in chronic infection

	1mpi	3mpi	5mpi	7mpi	9mpi	11mpi	13mpi	15mpi	17mpi	24mpi	27mpi
Number of leukocytes/ 1 µl blood	9200	11800	18600	10300	8100	11000	11300	16000	12900	9100	11200
% of atypical lymphocytes	6%	3.8%	9%	13%	3.5%	10.5%	14%	15%	8%	13%	8%

Atypical leukocytes observed in MHV-76 chronic infected mice could be classified as lymphoblasts (developing stages during maturation of neutrophile polymorphonuclear leukocytes)

antibodies and the maximum titre in virus neutralisation assay (titre 64) was found in the 10th month p.i. During 27 months of MHV-76 infection, BALB/c mice did not develop tumours. Connection between infection and tumour development was not confirmed.

Discussion

During the acute phase of infection we found MHV-76 and its antigen in many organs: the blood, thymus, lungs, heart, liver, spleen, bone marrow, peritoneal macrophages, lymph nodes, kidneys, brain and small intestine, but on a very low level in comparison with the prototype strain and other MHV isolates (Mistríková et al. 1994, 2002; Mrmusová-Šupolíková et al. 2003; Pappová et al. 2004). The primary site of viral multiplication were the lungs with the virus spreading to other organs of the lymphatic system by haematogenous route. The MHV-76 strain was eliminated from lungs already on days 5 - 7 p.i. similarly as described by Macrae (2001), but on day 10 p.i. the BALB/c mice infected with MHV-76 did not show apparent symptoms of illness in the course of acute infection, while 48% of 6-week-old mice infected with the prototype strain MHV-68 developed clinical signs and symptoms of illness at that time (Sunil-Chandra et al. 1992). We detected the highest infectious titre of MHV-76 during acute infection between days 5 and 7 p.i. in lungs in comparison with other MHV-isolates, reaching the highest titre in lungs on days 10 - 14 - 28. p.i. (Mistríková et al. 1994, 2002; Mrmusová-Šupolíková et al. 2003; Pappová et al. 2004). Intranasal inoculation with MHV-76 also lead to an initial lung infection, but in comparison with other MHV-isolates, the virus titre was decreased. We detected a very low level of virus in other organs, too. The titre of virus neutralising antibodies was low, although the MHV antigen as well as the infectious virus was detected at chronic intervals (ranging from 2 to 27 months) in all tested organs. Surprisingly, MHV-76 was found in the liver, brain and intestinal wall, later it was especially increased at 27 months p.i. In comparison with the prototype strain, lymphocytosis was very low and splenomegaly was not observed at all. Significantly reduced splenomegaly after MHV-76 infection was also described by Macrae et al. 2001. This time it was accompanied with a mild increase of leukocytes and very low percentage of atypical lymphocytes. We detected the virus in mammary glands during chronic infection with the maximal titre on 11 and 13 months p.i. These findings also indicate the possibility of secretion of the virus by maternal milk similarly to EBV (Junker et al. 1991). EBV-induced infectious mononucleosis (IM) is a benign self-limited lymphoproliferative disease characterised by expansion of lymphoid cells into atypical lymphocytes (Tomkinson et al. 1987). We observed the presence of atypical lymphocytes (leukocytes) in the blood first in immunocompetent mice infected with MHV-72 (Mistríková and Mrmusová 1998) and later in T-cell-deficient nude mice (Rašlová et al. 2000). Atypical leukocytes observed in the MHV-72 infected mice could be classified as lymphoblasts (developing stages during maturation of neutrophil polymorphonuclear leukocytes) similarly as in patients with myeloid leukaemia. Also

these atypical mouse leukocytes were highly similar in shape to those observed in patients suffering from infectious mononucleosis (Mistríková and Mrmusová 1998). In comparison with all MHV-isolates, MHV-76 caused very low levels of atypical leukocytes like IM or like the leukaemia syndrome (Mistríková et al. 2004). Persistence of the virus in many organs confirms the affinity of MHV-76 not only to cells of the lymphatic system, but also to neural cells (brain) and to glandular cells (mammary glands). Clambey et al. (2002) demonstrated that M1, M2, M3 and M4 genes and viral t-RNA-like genes are not essential for latency. A virus lacking these genes exhibits tissue-specific and route-of-infection dependent alterations in latency and reactivation. In the chronic phase BALB/c mice developed tumours after MHV-78 (7%), MHV-68 (11%), MHV-72 (13%), MHV-Šumava (14.6%) infection. In comparison with oncogenic MHV-strains, the chronic phase of infection with deletion mutant was not connected with tumour development. The presented results proved that MHV-76 is a lymphotropic virus, which has deletion of genes important in pathogenesis, namely in latency. And since the establishment of latency is needed for transformation with gammaherpesviruses, MHV-76 is a very important model for the study of latency and oncogenesis. During 27 months of infection with MHV-76 BALB/c mice did not develop tumours or the leukaemia-like syndrome. This is in contrast with MHV isolates, where latency was associated with lymphoproliferative disorders, lymphomas and even sarcomas in frequency of 7 to 22%. Works of Townsley et al. (2004), Evans et al. (2006) and Geere et al. (2006) were concerned with the role of the M4 gene, but none of these publications studied the effect of the M4 gene on long-term infection or its relation to tumour development. We hypothesize that the M4 gene, presented in all MHV-oncogenic isolates, may be related (directly or indirectly) to their transforming properties.

Patogenetická charakterizácia prirodzeného 9.5-kilobázového delečného mutanta (MHV-76) lymfotropného myšacieho gamaherpesvírusu 68

Myšací gamaherpesvírus 4 (MuHV-4) predstavuje zvierací model na štúdium živočíšnych gamaherpesvírusov. MHV-76 je prirodzeným delečným mutantom prototypového kmeňa MuHV-4 (MHV-68). MHV-76 chýba aspoň 12 otvorených čítacích rámcov na 5´ konci vrátane génov M1, M2, M3, M4 a osem vírusom kódovaných tRNA-podobných génov. 27 mesačná experimentálna infekcia BALB/c myší bola sledovaná po patogenetickej, imunologickej a onkogenetickej stránke. Po intranazálnej infekcii s MHV-76 bol infekčný vírus detegovaný v krvi, týmuse, pľúcach, srdci, pečeni, slezine, kostnej dreni, peritoneálnych makrofágoch, lymfatických uzlinách, obličkách, mliečnych žľazách, mozgu a tenkom čreve. Akútna fáza infekcie bola atenuovaná, ale chronická infekcia bola sprevádzaná dlhotrvajúcou perzistenciou vírusu nielen v lymfatických, ale aj v nervových a glandulárnych tkanivách. Pri porovnaní s prototypovým kmeňom bola splenomegália a lymfocytóza veľmi mierna. Počas 27-mesačnej infekcie BALB/c myší s MHV-76 sa prekvapivo neobjavilo lymfoproliferatívne ochorenie podobné infekčnej mononukleóze, leukémia ani lymfómy. Predpokladáme, že M4 gén, vyskytujúci sa u všetkých onkogénnych MHV izolátov, by mohol byť zapojený (priamo alebo nepriamo) do ich transformujúcich vlastností.

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