A MURINE GAMMAHERPESVIRUS

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Summary. – In 1976, within a project on isolation of herpesviruses from small rodents in former Czechoslovakia, the mouse herpesvirus strain 68 (MHV-68) was isolated (Blaškovič *et al.*, 1980). This virus was accepted by *The International Committee on Taxonomy of Viruses* (ICTV) as a new, so far unassigned species (member) of the *Gammaherpesvirinae* subfamily of the *Herpesviridae* family (Murphy *et al.*, 1995). Besides MHV-68, four more isolates (MHV-60, MHV-72, MHV-76, and MHV-78) similar to MHV-68 were obtained in that field experiment in Slovakia. Later, three more isolates (MHV-Šumava from Bohemia and MHV-4556 and MHV-5682 from Slovakia) were obtained in other field experiments. All these isolates are in some properties similar but in others different from each other. Nevertheless, as their comparative genome sequence analysis is not yet available, we propose at present to regard all the abovementioned isolates as different isolates of the same virus and strain, i.e. MHV-68. It is not excluded that a more detailed characterization of these isolates in the future will lead to proposals of designating some of these isolates as new strains of the virus of concern. This review summarizes the up to date knowledge of various biological and physico-chemical properties of MHV-68. At least three isolates, MHV-68, MHV-72 and MHV-Šumava seem to be involved in malignant neoplasm development in mice. It should be stressed that the pathogenesis of the induced lymphoproliferative disease in mice is similar to that caused by Epstein-Barr virus (EBV) in humans.

Key words: mouse herpesvirus strain 68 (MHV-68); murine gammaherpesvirus; MHV-60, MHV-72, MHV-76, MHV-78, MHV-4556, MHV-5682, MHV-Šumava; characterization; Epstein-Barr virus; lymphoproliferative disease; pathogenesis

Introduction

It has been generally confirmed that viruses of the *Herpesviridae* family occur in both warm- and cold-blooded vertebrates and invertebrates. There have been more than a hundred species of herpesviruses described so far. Every year the isolation of a new herpesvirus from different animals is being reported. It is perhaps not exaggerated that each animal species on the Earth hosts its own species of

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Abbreviations: ACV = acyclovir; AIDS = acquired immunodeficiency syndrome; AIHV-1 = alcelaphine herpesvirus 1; AMC = adherent mononuclear cell; AZT = azidothymidine; BoHV-4 = bovine herpesvirus 4; CCPH = complement control protein homologue; CF = complement fixation; CPE = cytopathic effect; EBER = EBV-encoded RNA; EBV = Epstein-Barr virus (human herpesvirus 4); EHV-2 = equine cytomegalovirus 2; FUDR = fluorodeoxyuridine; GCV = gancyclovir; gp = glycoprotein; HVS-2 = herpesvirus saimiri 2; HSV-1, HSV-2 = herpes simplex viruses 1 and 2 (human herpesviruses 1 and 2, HHV-1 and HHV-2); i.c. = intracerebral(ly); IF = immunofluorescence; IFN = interferon; IL-8 = interleukin 8; i.n. = intranasal(ly); i.p. = intraperitoneal(ly); i.v. = intravenous(ly); KSHV = Kaposi's sarcoma-associated herpesvirus; LPD = lymphoproliferative disorder; MCMV-1 = mouse cytomegalovirus 1; MHV-68 = mouse herpesvirus strain 68; MuMG = normal murine mammary glands; VN = virus neutralization; OHV-1 = ovine herpesvirus 1; ORF = open reading frame; p.i. = post infection; p.o. = peroral; RCA = regulators of complement activation; RCMV = rat cytomegalovirus; REF = rabbit embryo fibroblast; TK = thymidine kinase; TNF = tumor necrosis factor

herpesviruses. The *Herpesviridae* family is divided into three subfamilies: *Alpha-, Beta- and Gammaherpesvirinae*. Gammaherpesviruses are divided into two genera: *Lymphocryptovirus* and *Rhadinovirus*. The host range of gammaherpesviruses is frequently but not exclusively limited to the family or to the order to which the natural host belongs. *In vitro,* all gammaherpesviruses replicate in lymphoblastoid cells and some of them also cause lytic infection in certain types of epitheloid and fibroblastic cells. These viruses tend to be specific for either T or B lymphocytes, but exceptions do occur. In the lymphocyte, the infection often proceeds without the production of infectious virus progeny. The virus in latent state is frequently demonstrated in lymphoid tissues. The interest in the biology of the gammaherpesviruses stems largely from their association with a variety of malignancies.

Gammaherpesvirus family members infect various animals like mice, rabbits, wildbeest, sheep, cattle, horses and primates (humans, macaques, monkeys, baboons etc.) EBV was described as a cause of benign diseases as infectious mononucleosis (glandular fever) and oral hairy leukoplakia and is associated with several malignancies as Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease. Kaposi´s sarcoma-associated herpesvirus (KSHV) was found as an essential cofactor of sarcoma, Castleman's disease and primary effusion lymphoma in acquired immunodeficiency syndrome (AIDS) patients. Herpesvirus saimiri 2 (HVS-2) is non-pathogenic for its natural host. Similarly, alcelaphine herpesvirus 1 (AIHV-1) is non-pathogenic for its natural host but causes a fatal disease, malignant catarrhal fever in various ruminants.

Little is known that MHV-68, an unassigned species of the subfamily *Gammaherpesvirinae* (Murphy *et al.*, 1995) is only one of several herpesvirus isolates obtained from murine rodents trapped at the territory of former Czechoslovakia. All the isolates from Slovakia from 1980 (MHV-60, MHV-68, MHV-72, MHV-76, and MHV-78) as well as three more isolates obtained in 1985 or later (MHV-Šumava from Bohemia and MHV-4556 and MHV-5682 from Slovakia) are antigenically highly similar. At least three isolates, MHV-72, MHV-Šumava, and MHV-68, seem to be involved in malignant neoplasm development. MHV-68 draws much attention providing a valuable animal model for exploring the interaction of gammaherpesviruses with their natural hosts, the nature of EBV infection *in vivo*, the genesis of the EBV-associated diseases as well as the host response to a gammaherpesvirus infection in humans.

Isolation

In November 1976, during a field work on the ecology of arboviruses on the territory of Slovakia, close to the city of Bratislava, small rodents were trapped and investigated for the presence of herpesviruses. Newborn laboratory mice were inoculated intracerebrally (i.c.) with pooled 10% suspensions from different organs (lungs, spleen, heart, liver and kidney) of the exsanguinated rodents. Suspensions from two species of murine rodents, *Apodemus flavicollis* and *Clethrionomys glareolus*, were tested. The inoculated mice developed after the 1st , 2nd or 3rd successive mouse-to-mouse i.c. passages severe or fatal encephalitis. Brain suspensions prepared from the succumbed mice were inoculated into BHK-21 and rabbit lung ZP cell lines, which developed a cytopathic effect (CPE). Within 3-5 days p.i. a CPE occurred resembling that caused by herpesviruses, namely the cell rounding, swollen nuclei and the Cowdry A type intranuclear inclusions (Blaškovič *et al.*, 1980). From the infected cells, altogether five isolates were obtained (MHV-60, MHV-68, MHV-72, MHV-76, and MHV-78). MHV-68 was twice purified by limiting dilution and the MHV-68 clone g2.4 was chosen after propagation in BHK-21 cells for further characterization (Efstathiou *et al.*, 1990a,b). Later, all the five isolates were repeatedly plaque-purified and double agar layer technique (M. Stančeková, unpublished data) and finally propagated in BHK-21 and/or Vero cells. MHV-72 was propagated in Vero (Mistríková *et al.*, 1994) or murine mammary gland (MuMG) cells to titers up to 10^9 PFU/ml (Rašlová *et al.*, 2000a,b). Another isolate, MHV-Šumava was obtained from the lungs of *Apodemus flavicollis* that showed serum antibodies to MHV-68. It was passaged in rabbit embryo fibroblasts (REFs) reaching titers up to 10^9 $TCID_{50}$/ml in the 13th passage and causing a typical CPE from the 2nd passage (Mistríková and Blaškovič, 1985). Later, it was repeatedly plaque-purified (M. Stančeková, unpublished data). Two further isolates, MHV-4556 and MHV-5682 were obtained from the brain of *Apodemus flavicollis* and purified in the same way.

Growth characteristic

In contrast to most gammaherpesviruses, the MHV-68 isolates cause a productive infection in conventional cell monolayer culture. Twenty cell lines of different origin (bird, rodent, carnivore, pig, monkey, and man) were able to propagate two different isolates (Table 1) reaching titers ranging from 10^2 to 10^7 $TCID_{50}$/ml. The growth curves of MHV-60, MHV-68, MHV-72, MHV-76 and MHV-78 resembled those obtained with HSV-1, HSV-2, pseudorabies virus and guinea pig herpesvirus 1 (Hsiung-Kaplow herpesvirus) in the identical cell line. The MHV-68 isolates persisted in cell lines of B lymphocyte origin. Furthermore, MHV-68 established a persistent infection in mouse myeloma cells and NS0 cell line (Sunil-Chandra *et al.*, 1993). Besides mouse B cell lines (SP2/0 and NS0) also mouse peritoneal macrophages were found to be permissive for the MHV-68 isolates, namely MHV-72 (Mistríková *et al.*, 1994).

Table 1. Growth of MHV-68 and MHV-72 in various cell cultures

Cell culture (line)	Origin	No. of blind passages	No. of positive passages	Maximum infection titer ($TCID_{50}$ /ml)	Type of CPE (day p.i.[a])
CEC	Chick	3	7	2.0	CR (3)
L	Mouse	2	30	5.5	CR (2.2)
MEF	Mouse	0	1	3.5	CR (3.5)
NMuMG	Mouse	0	40	9.0	CR (3.5)
RAT	Rat	0	20	4.5	CR (3)
SIRC	Rabbit	0	3	3.0	CR (3.75)
ZP	Rabbit	2	30	3.0	CR (3.5)
REF	Rabbit	0	50	7.0	CR (2.5)
BHK-21	Hamster	1	15	6.0	CR (4.5)
GPT	Guinea pig	0	20	5.5	PKC (5.1)
CCL-64	Mink	0	3	2.0	CR (3)
PS	Swine	0	5	4.0	CR (5)
GMK	Monkey	0	5	3.0	CR (2.5)
Vero	Monkey	0	100	7.0	CR (3.5)
Am-57	Human	0	3	3.0	CR (4)
HeLa	Human	2	5	2.0	CR (4)
LEP	Human	2	5	2.0	CR (3.5)

[a]Average values.
CR = cell rounding, PKC = polykaryocytes.

Morphological properties

Electron microscopy of REFs infected with MHV-60, MHV-68, MHV-72, MHV-76, or MHV-78 (Blaškovič *et al.*, 1980) revealed similar morphological changes characteristic for herpesvirus infection. However, the formation of intranuclear tubular structures was more frequent with MHV-72 and MHV-78 as compared to the formation of intracytoplasmic dense bodies with MHV-78. The ultrastructural changes in the cells caused by all the isolates resembled those caused by freshly isolated HSV-1, but some morphological changes resembled those characteristic for cytomegaloviruses (Čiampor *et al.*, 1981).

Serological characteristic

Complement fixation (CF) and virus neutralization (VN) tests confirmed that MHV-60, MHV-68, MHV-72, MHV-76, and MHV-78 are antigenically identical or very closely related. An immunofluorescence (IF) test showed that the antigen assembly and distribution within the infected cell resemble those of alphaherpesviruses. A radioimmunoassay revealed close antigenic relatedness among the five isolates mentioned above (Svobodová *et al.*, 1982b). Furthermore, MHV-4556 and MHV-5682 were found to cross-react with MHV-72 in an immunoprecipitation assay (Kožuch *et al.*, 1993). In an IF test, MHV-Šumava cross-reacted with MHV-68 as well as MHV-72 (J. Mistríková, unpublished data).

Genome

First data concerning MHV-Šumava genome were reported by Blaškovič *et al.* (1988). It was shown that the DNA genome has a size of 135 kbp and a GC content of 60%. According to these preliminary results this isolate was considered an alphaherpesvirus. With regard to later data obtained by complex characterization of MHV-68 genome we can assume that the genome size and GC content vary among different isolates containing different number of terminal repeats. The characterization of another isolate, MHV-76 is so far also incomplete. Restriction profiles of MHV-76 DNA obtained with *Bam*HI, *Eco*RI, *Hind*III and *Pst*I were compared with corresponding profiles of DNAs of two rodent herpesviruses – mouse cytomegalovirus (MCMV) and rat cytomegalovirus (RCMV). On the basis of close similarity of MHV-76 and RCMV restriction profiles as well as their homology found in hydridization experiments MHV-76 was regarded as a RCMV strain infecting mice and thus a betaherpesvirus (Hamelin *et al.*, 1992). However, more recent results confirmed that the both abovementioned papers characterized the virus genome not enough for classification purposes. Detection of MHV-68 in experimentally infected mouse thymocytes controversially supported the idea that this virus is probably a gammaherpesvirus (Rajčáni *et al.*, 1985). Furthermore, the analysis of polypeptides present in the nuclei and cytoplasm of the MHV-72-infected cells (Reichel *et al.*, 1994) and the comparison of proteins specified by various isolates (MHV-68,

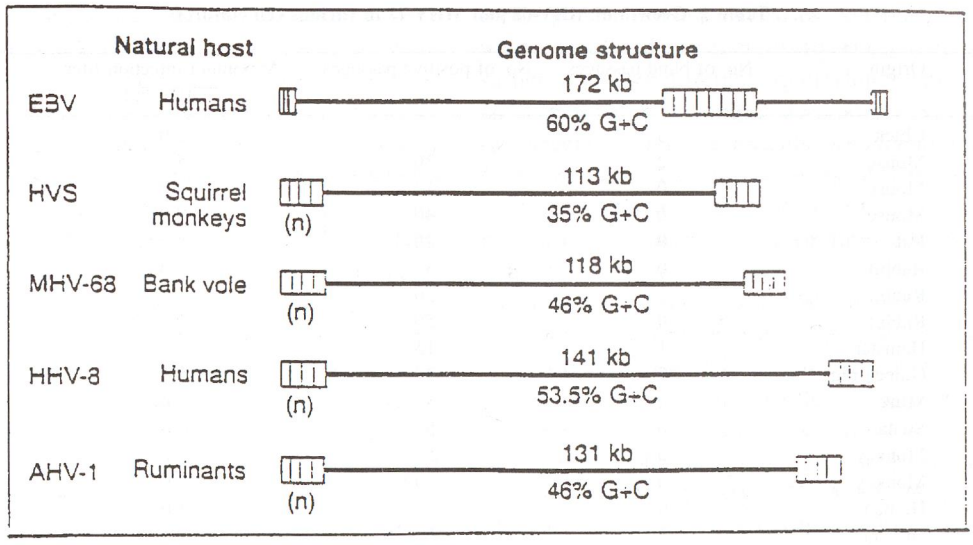

Fig. 1
Genome organization of some gammaherpesviruses
The genome of all these viruses contains terminal repeats. A central unique region of various size (113–141 kbp) is continuous in all these viruses except EBV which contains also internal repeats (Simas and Efstathiou, 1998).

MHV-72, and MHV-76) (Reichel *et al.*, 1991) indicated no obvious homology to alphaherpesviruses or betaherpesviruses.

Later results based on the construction of restriction maps and sequencing of MHV-68 genome definitely confirmed that its structure is typical for a gammaherpesvirus. MHV-68 genome has been intensively studied since 1990 when its restriction map was published and the library of the most restriction fragments was prepared (Efstathiou *et al.*, 1990a). Recent restriction mapping of MHV-72 confirmed that MHV-68 and MHV-72 are very close but distinct (M. Kúdelová, unpublished data).

The unique sequence of MHV-68 DNA is 118.2 kbp long and flanked with variable multiple copies of a 1.23 kbp terminal repeat. It is not known whether the number of terminal repeats is constant. These repeats probably function in the cleavage and packaging of the genomic length DNA. The organization of MHV-68 genome is characteristic for gammaherpesviruses. Sequencing of seven restriction fragments of the unique MHV-68 DNA sequence revealed nine open reading frames (ORFs) and confirmed the colinearity of genomes of MHV-68 and gammaherpesviruses as EBV and more closely HVS-2 (Efstathiou *et al.*, 1990b).

A total of 17 ORFs, one of which was spliced [BKRF2 + BKRF3 – M transactivator (EB2 in EBV) homologue] were identified and analyzed (Mackett *et al.*, 1997). Comparison to other gammaherpeviruses showed that MHV-68 is marginally closer to HVS-2 and equine cytomegalovirus 2 (EHV-2) than to EBV. It was disappointing that none of the unique EBV genes except probably the gp150 gene was present in this 20 kbp sequence in MHV-68 which was considered a model for EBV. Nevertheless, temporal regulation of these genes was found to be similar to homologous genes in EBV. Moreover, transcripts of two early genes not found in EBV were observed (dUTPase and helicase/primase). Sequence analysis of the 6,162 kbp long *Hind*III E restriction fragment on the left of MHV-68 genome brought an exciting discovery. Besides two unique ORFs (ORF2 and ORF3 later designated as M2 and M3) one – ORF1 (M1) – found in this locus of the genome displayed homology to those of the poxvirus proteins of the serpin family (Smith *et al.*, 1989). The protein encoded by ORF1 (M1) does not appear to have an active site region characteristic for poxvirus serpins and whether it regulates apoptosis or controls host inflammatory responses is so far unknown (Bowden *et al.*, 1997). Moreover, near the left end of the DNA molecule, upstream of ORF1 (M1) and interspersed within ORFs 1–3 (M1–M3), the sequences coding for eight novel small RNAs were found. They have a characteristic of transfer RNA and a significant homology to bacterial tRNAs (Bowden *et al.*, 1997). Transcripts of these tRNAs (designated as viral tRNAs–vtRNAs) are processed into mature tRNAs and at least for four of them it was demonstrated that they are not charged. *In vivo*, they could be first detected in splenic tissue of infected mice at day 7 post infection (p.i.) and then as late as at day 70 p.i. (Simas *et al.*, 1999). tRNA-like genes are abundantly

expressed during a lytic infection of BHK cells and within the splenic germinal centers of latently infected mice; they could be used as a marker of latent infection. Recently, it was estimated that the deletion of tRNA-like sequences 1–4 and ORF M1 did not affect the ability of MHV-68 to establish latency and to reactivate from it (Simas and Efstathiou, 1998). Thes are the first tRNA-like sequences encoded by a virus of eukaryotes which were detected though functional tRNA genes have been previously described in bacteriophages T4 an T5 (Calendar, 1988). Their biological function remains currently unclear. Similar non-tRNAs maintaining a tRNA-like structure were found in some plant mosaic viruses (Dreher *et al.*, 1996). As regards their position in the genome, analogous to the MHV-68 tRNAs-like sequences are HVS-2 HSUR genes expressed in transformed T cells (Murthy *et al.*, 1989). In addition, EBV-encoded RNA (EBER) which is situated at the opposite terminus of EBV genome and is characteristic of the EBV latency could represent an analogue to MHV-68 tRNAs (Swaminathan *et al.*, 1992). Though the small RNAs of gammaherpesviruses are structurally dispersed their detection in a significant portion of cells could prove the presence of latent virus within the spleens of latently infected animals.

MHV-68 DNA sequence

In 1997, the complete genomic sequence and the deduced *Bam*HI, *Eco*RI and *Hind*III restriction maps of MHV-68 with the ORFs and tRNAs-like genes were published by Virgin *et al.* (1999) (GenBank Acc. No. U 97553). The virus genome represented by a linear double-stranded DNA was shown to consist of 118,237 bp of unique sequence (with a GC content of 46%) flanked by multiple copies of a 1,213 bp long terminal repeat (with a GC content of 78%). More than 80 gene products are encoded by the unique portion of the genome which is largely colinear with those of KSHV, HVS-2, and EBV. Because 63 ORFs were found to be homologous to HVS-2 and KSHV, the HVS/KSHV numbering system was used to define these ORFs. Comparative analysis of the MHV-68, HVS-2, EBV and KSHV genomes demonstrated that each of them has large colinear gene blocks interspersed by regions containing virus-specific ORFs. The unique region of the MHV-68 genome was found to have two types of internal repeats: a block of five 40-bp-long repeats (each containing a *Bam*HI restriction site) downstream of nt 26,778 and a block of two 100-bp-long repeats downstream nt 98,981.

MHV-68 unique ORFs

The knowledge of the MHV-68 genome sequence enabled to compare it with that of HVS-2 using ORF numbers corresponding to HVS-2 gene numbers to show the conserved blocks of genes. The ORFs largely unique to MHV-68 were designated as M, those largely unique to HVS-2 as S, and those largely unique to KSHV as K (Fig. 2). Similarly, largely unique ORFs observed in two further fully sequenced gammaherpesvirus genomes were designated as A (for AIHV-1) and E (for EHV-2).

Besides ORFs homologous to those of HVS-2 and KSHV [e.g. a complement regulatory protein (ORF4), a D-type cyclin (ORF72), a G protein-coupled receptor with homology to interleukin 8 (IL-8) receptor (ORF74)], a great attention drew 16 ORFs predicted to encode proteins unique to MHV-68 – ORFs designated M1 to M16.

Recently, a similarity between M1 and M3 gene sequences as well as between their putative protein products was found. It seems that these genes arose from a duplication event (van Berkel *et al.*, 1999).

Eleven MHV-68 ORFs were studied in the group of candidates for latency-associated genes by Virgin *et al.* (1999). Their transcripts were detected in the spleen and peritoneal cells of latently infected B cell-deficient MuMT mice containing high levels of reactivable latent MHV-68 (Weck *et al.*, 1996). These authors suggested that probably different genes are expressed during latency in different mouse organs. Three various regions in M2, M11, ORF73 and ORF74 were proved to be transcribed during latent infection and were chosen as markers for detection of latency. On the other hand, M3 and M9 were abundantly transcribed during lytic infection as well as latency. By analogy to EBV latent transcripts, it is possible that these latent transcripts span lytic genes in M3 and M9. Only two regions – ORFs 73 and M11 – were found to be homologous to the genes transcribed during EBV and/or KSHV latency. None of MHV-68 unique M4–M8 was significantly expressed in either the latently infected spleen or peritoneal cells. Further MHV-68 unique ORFs, M10 (a, b, and c) and M12–M14 spanning the GC-rich 100 bp repeat region or GC-rich terminal repeats were not screened (Virgin *et al.*, 1999).

Three of unique MHV-68 ORFs, M2, M3, and M9 encode putative products with no clear homologues in the GenBank database. During latency *in vitro* and *in vivo* but not in lyticaly infected fibroblasts, the M2 gene expression was confirmed by other authors (Husain *et al.*, 1999). The M2 transcript has two exons and a protein product of expected size with a CD8+T cell epitope. M2 protein is also expressed in S11 cell line derived from splenocytes of Balb/c mice harvested 24 days p.i. M2 protein is likely linked to MHV-68 latency. After recognizing the epitope of concern in MHV-68 infected mice, the M2 protein is considered a target for the host cytotoxic T lymphocyte response.

In the spleen of infected Balb/c mice the expression of M3 could be detected on day 21 p.i. The other 19 ORFs

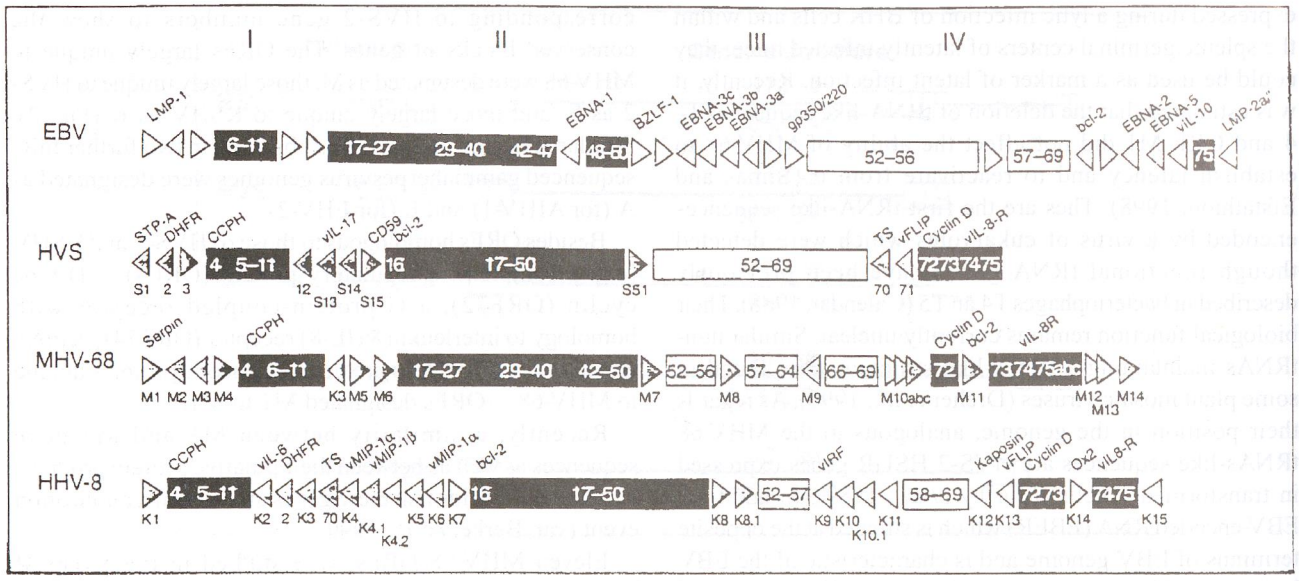

Fig. 2

Gene structure of some gammaherpesviruses

The EBV genome is inverted relative to its conventional orientation. EBV genes with no homology to those of others under comparison: EBNAs (EBV nuclear antigens), LMP1 and LMP2 (latent membrane proteins 1 and 2), and BZLF-1 (EBV-encoded transactivator). Conserved blocks I, II, III, and IV and the ORFs indicated within these blocks relate to the HVS-2 numbering. Between the gene blocks are interspread ORFs which are largely unique for each virus and are designated as S (HVS-2), M (MHV-68), and K (HHV-8).

bcl-2 = B cell lymphoma gene; DHFR = dihydrofolate reductase; GCR = G protein-coupled receptor; STP-A = saimiri transformation protein A; TS = thymidylate synthetase; vIL-6, vIL-17 = viral interleukins 6 and 17; vIL-8R = viral IL-8 receptor; vIRF = viral IFN-responsive factor; vFLIP = viral FLICE (caspase 8) inhibitory protein; vMIP-1 a/b = viral macrophage inflammatory protein 1 a/b (Simas and Efstathiou, 1998).

tested (M1, M2, M4, ORF4, K3, M7, M8, M10 (a, b, and c), ORF72, M11, ORF73, ORF74, and M12–M14) were not expressed (Simas *et al.*, 1999). Furthermore, M3 transcripts were detected up to day 46 p.i. in latently infected splenocytes isolated from B cell-deficient mice (van Berkel *et al.*, 1999). The M3 mRNA is unspliced and the putative M3 product is a secreted protein of 45K with an amino-terminal signal sequence (GenBank Acc. No. AF 127083). Indeed, M3 protein was abundantly secreted into the medium from MHV-68 infected cells. M3 protein expressed during the establishment of latency might play a role in modulating the host immune response to MHV-68 infection.

Another MHV-68 unique ORF, M7 was characterized as the first of the M ORFs. It encodes glycoprotein (gp) 150 (gp150) (Stewart *et al.*, 1996) which currently serves as a marker for late lytic MHV-68 infection.

Conserved MHV-68 ORFs

Comparing to other gammaherpesviruses (using the HVS-2 numbering system) the conserved MHV-68 genes could be divided into four gene blocks: I – from ORF4 to ORF11; II – from ORF17 to ORF50; III – from ORF52 to ORF69, and IV – from ORF72 to ORF75 (Fig. 2). Interspersed within these blocks are largely unique MHV-68 ORFs described earlier. From the conserved MHV-68 genes there is one exception – K3 gene is not present in the abovementioned blocks but is homologous to HVS-2 ORF12, KSHV K3 and K5 genes and BoHV-4 IE gene 1. The latter genes contain conserved sequences related to a zinc-finger-like motif that most likely indicates a common evolutionary origin (Simas and Efstathiou, 1998).

Recently, MHV-68 gene 50, a single immediate early gene that appears to be conserved among the so far characterized gammaherpesviruses was described. It was demonstrated that MHV-68 gene 50 plays pivotal role in regulating virus reactivation. It is transcribed to both spliced and unspliced mRNAs. The predominant spliced mRNA of the gene 50 extends upstream of the ORF by encoding an additional 94 amino acids long sequence thus extending the amino-terminal sequence. The predicted amino-terminal end of the gene 50 product encoded by the spliced mRNA shows significant homology to the gene 50 products encoded by HVS-2, KSHV and EBV (Liu *et al.*, 2000). It is interesting that for HVS-2, KSHV and MHV-68 the extending of the gene 50 ORF requires the presence of the spliced mRNA,

while in EBV both the spliced and unspliced mRNAs of the gene 50 (BRLF1) are predicted to encode the same product. The MHV-68 gene 50 encoded protein is expressed at low levels but reaches higher levels in the presence of ongoing viral infection. This protein could transactivate the MHV-68 gene 57 promotor which has a very low basic activity. In other gammaherpesviruses, the gene 57 promotor was shown as a target of the gene 50 encoded transactivator too (Lukac *et al.*, 1999). Furthermore, the region upstream of the putative MHV-68 gene 50 transcription initiation site was demonstrated to have orientation-dependent promotor activities. Thus a detailed study of the MHV-68 gene 50 including its similarities/dissimilarities to corresponding genes of other gammaherpesvirus could help to understand better the establishment of viral latency and the virus reactivation.

A phylogenetic trees depicting three subfamilies of herpesviruses (alpha-, beta- and gamma-herpesviruses) were constructed using the conserved gene sequences, especially the genes encoding gpB (MHV-68 ORF8) and DNA polymerase (MHV-68 ORF 9) (McGeoch *et al.*, 1995; Damania *et al.*, 2000).

MHV-68 ORFs with cellular counterparts

In MHV-68 genome, four ORFs (M11, ORF72, ORF4, and ORF74) with clear cellular counterparts and/or anti-apoptotic function were detected.

M11 was the first ORF of MHV-68 to which a cellular counterpart was found similarly to those of other gammaherpesviruses. The M11 sequence has some similarity to bcl-2 family members encoding a protein which presumably has one of the two conserved domains, BH1, but apparently lacks the BH2 domain (Wang *et al.*, 1999). On the other hand, Wang *et al.* (1999) found that M11 protein (v-bcl-2) was expressed during lytic but not latent infection with MHV-68 and was localized predominantly in the cytoplasm. It was shown that M11 protein is able to protect MHV-68-infected cells from destruction by blocking the apoptosis induced by Fas and tumor necrosis factor (TNF) allowing the virus to complete its replication cycle and/or to promote the survival of cells destined for latency.

A viral cyclin (v-cyclin), a protein of 25 K encoded by MHV-68 ORF72, is homologous to mammalian D-type cyclins (van Dyk *et al.*, 1999). Similar v-cyclins were identified in other gammaherpesviruses (e.g. HVS-2 and KSHV). However, EBV does not encode such cyclin homologue. ORF72 is expressed with leaky-late kinetics in lytically infected cells and was demonstrated to promote the cell cycle progression in primary lymphocytes. In concordance with the predicted ability of v-cyclins to regulate cyclin-dependent kinase/s and thus to promote cellular proliferation, the expression of MHV-68 v-cyclin caused lymphoblastic lymphoma in v-cyclin-transgenic mice. These results identified MHV-68 v-cyclin as an oncogene in the absence of other viral gene products. However, one or more additional gene products are considered to be required to protect cells from apoptosis. Collaboration of MHV-68 v-cyclin (ORF72) and v-bcl-2 (M11) is probably involved in this processes resulting in promotion of lymphocyte proliferation and acceleration of tumor development (van Dyk *et al.*, 1999).

Primate gammaherpesviruses KSHV and HVS-2 share an ORF predicted to encode a protein structurally related to host regulators of complement activation (RCA). In MHV-68, ORF4 was found to encode RCA protein strikingly homologous to a human decay-accelerating factor (Kapadia *et al.*, 1999). ORF4 located between M4 and ORF6 is transcribed into a late bicistronic mRNA. Although the transcripts of the corresponding MHV-68 and HVS-2 genes are different, in both cases multiple isoforms of RCA protein are expressed. The soluble as well as membrane-bound MHV-68 RCA proteins (M_r of 40–45 K and 60–65 K, respectively)) have short cystein-rich consensus repeats which are critical for binding C3b and C4b and inhibit murine complement activation regulating both classical and alternative pathways. Similar functions were found for HVS-2 CCPH and/or HSV-2 gC which protect cells from lysis in VN and CF tests. Thus MHV-68 RCA protein could play a specific role in pathogenesis by protecting virions and/or virus-infected cells from a complement-mediated damage (Kapadia *et al.*, 1999).

MHV-68 ORF74 was shown to encode an IL-8 receptor (a G protein-coupled receptor) homologue and to have its counterpart in KSHV ORF74. The G protein-coupled receptor is a viral oncogene which can induce transformation and angiogenesis in KSHV-mediated oncogenesis (Bais *et al.*, 1998). Simas *et al.* (1999) confirmed that ORF74 is not expressed during latent infection with MHV-68. On the contrary, ORF74 was found together with ORF73 within one of three transcriptionally active regions during latency and was chosen as a marker of latency (Virgin *et al.*, 1999). Characterization of MHV-68 ORF74 protein as a further potential viral oncogene is currently in progress.

MHV-68 glycoproteins

Glycoprotein B (gpB) encoded by ORF8 is located in the cytoplasm and nuclear margins but not on the surface of infected cells or in virions. It is produced late in the virus replication cycle. gpB (105 K) has 2 hydrophobic domains and 9 potential N-linked glycosylation sites. MHV-68 gpB is most similar to EBV gpB (gp110). gpB antibodies do not neutralize the virus infectivity. MHV-68 gpB like EBV gpB functions in virus assembly and/or egress (Stewart *et al.*, 1994). Unlike the abovementioned gBs, the gpB of bovine

herpesvirus 4 (BoHV-4) is a major component of the virion (Lomonte *et al.*, 1997).

Ttransmembrane gp150 is present on the surface of infected cells and is incorporated into virions. It is encoded by ORF M7 earlier designated as BPRF1. Besides a large major protein (150 K) two smaller minor proteins (gp110 and gp130) were observed in MHV-68-infected cells. gp150 is probably the precursor of gp130. gp150 has high content of serine and proline as well as O-linked glycosylation sites. A region containing 22 proline-rich amino acid repeats is located within an ectodomain towards the membrane anchor (Stewart *et al.*, 1996). In the gp150 gene sequence of MHV-72 a mutation changing codon for amino acid 314 was found; it caused duplication of a 15 amino acids long repeat situated in the proline-rich region (K. Mačáková unpublished data). As gp150 antibodies neutralize the virus, gp150 represents a potential target for immune response (Stewart *et al.*, 1996). The homology of MHV-72 gp150 to EBV membrane antigen (gp340/220) suggested to use a recombinant vaccinia virus expressing gp150 as a model of EBV gp350-based vaccine protecting against virus-associated disease in man (Stewart *et al.*, 1999).

Glycoprotein H (gpH) was characterized together with thymidin kinase (TK). Genes of these proteins are located next to each other and have significant homology with corresponding genes of other gammaherpesviruses. gpH was detected in the envelope of all herpesviruses. MHV-68 gH encoded by ORF22 (BXLF2) is probably transcribed into a late bicistronic mRNA spanning ORF21 (encoding TK) too. An analogous transcript, with exception that it is transcribed early but not late, was detected in EBV. MHV-68 gpH structure resembles those of other gpHs in having a signal leader sequence at the N-terminus and a membrane anchor sequence at the C-terminus with predicted size of 82.8 K. MHV-68 gpH antibodies were shown to be virus neutralizing. Thus MHV-68 gpH plays probably a role in virus entry into target cells by fusing with their membrane (Pepper *et al.*, 1996).

MHV-68 thymidine kinase

MHV-68 TK gene (ORF21) was sequenced by Pepper *et al.* (1996) and the deduced amino acid sequence was aligned to those of TKs of 15 other herpesviruses. It showed homology in 6 conserved sites. MHV-68 TK was thought to be unique among other herpesvirus TKs within the consensus binding site (GXXGXGK) in which the middle glycine is replaced by alanine. The comparison extended to 9 other herpesvirus TKs using the GenBank database and ovine herpesvirus 1 (OHV-1) TK (V. Zelník, unpublished data) revealed two more gammaherpesviruses which have the abovementioned change in TK amino acid sequence (AHV-1 and OHV-1). Recently, MHV-72 TK gene was found to be identical to that of MHV-68 (Rašlová *et al*, 2000b). TK of both isolates has a long region of 235 amino acids upstream of the first conserved site, a feature typical for gammaherpesviruses. MHV-68 TK is more closely related to that of HVS-2 than to that of EBV. MHV-68 TK has a predicted size of 68.4 K and displays an *in vitro* functional TK activity (Pepper *et al.*, 1996). Cells infected with either MHV-68 or EBV were shown to be sensitive to acyclovir (ACV) demonstrating thus functional similarity of MHV-68 and EBV TKs (Sunil-Chandra *et al.*, 1994b). Recently, MHV-68 was used as an attractive model for the study of antiviral drugs. The usefulness of MHV-68 TK as a suicide gene for therapy was evaluated (Rašlová *et al.*, 2000b).

Pathogenetical characteristic

The natural route of MHV-68 infection in murine species is not known. However, based on the knowledge of other herpesvirus infections, it is likely that it involves a close contact, probably respiratory or peroral transmission. Therefore in an experimental context, peroral or intranasal infection represents the most authentic route.

Experimental pathogenesis of MHV-68 was first studied in newborn mice. They were found to be susceptible to peroral infection. Death as a consequence of encephalitis occurred within 6–8 days p.i. with doses over 10^5 $TCID_{50}$. Clinical symptoms also occurred in some animals infected with lower doses, while others developed inapparent infection as judged by the presence of humoral antibodies on day 60 p.i. MHV-68 was detected in the lungs, blood, liver, spleen, kidneys, heart muscle, brain and urinary bladder of sick animals. Necrotizing pneumonia accompanied the virus replication in the epithelial cells of alveolar ducts and alveolar lining as confirmed by IF test and histological examination. Latent infection of Gasserian ganglia in the survivors was not necessarily related to the administered virus dose (Blaškovič *et al.*, 1984). When outbred laboratory mice were inoculated at age of 5, 10 and 21 days by oral and /or i.n. routes with 2 different (lethal and non-lethal) doses of MHV-68, severe exudative pneumonia with hematogenous dissemination of the virus to liver, heart muscle and kidneys developed in the 5-day-old as well as in a part of the 10-day-old mice. The virus antigen was found by IF test in the alveolar linning of lungs, heart muscle fibers, spleen and thymic lymphocytes, tubular epithelial cells of kidneys, neurons of Gasserian ganglia and the intima of large pulmonary vessels. Electron microscopy confirmed the transfer of virus particles through the capillary endothelium of the damaged alveolar septa. MHV-68 was recovered both by direct examination of the tissue homogenates as well as by an explantation technique. The results are suggestive for a dynamic persistence of MHV-68 rather than for static latency (Rajčáni *et al.*, 1985). The degree of spread of MHV-68 to other tissues depends on the duration of infection and route used. In mice a viremia is observed for up to 3 weeks post i.n.

inoculation, in which various tissues become infected, notably adrenal glands, spleen, liver, kidneys and heart. The same pattern of infection is seen in older animals after i.p. or i.v. virus inoculation. In the lungs, the infected cell types include alveolar epithelial cells and mononuclear cells. The productive infection lasts for about 10 days before clearance by the immune system (Nash *et al.*, 1996). The absence of neural spread of MHV-68 was found provided the virus was inoculated into the right scarified cornea, but the virus spread to lungs and liver took place by haematogenous route. Later on, the virus was recovered from lungs, spleen and trigeminal ganglia. The virus isolation rate from trigeminal ganglia and brain stem increased considerably after explantation. It was confirmed that the persistence of MHV-68 is productive (Rajčáni *et al.*, 1987).

The pathogenesis of MHV-68 was compared to that of MCMV. One-, 10-, and 21-day-old and adult mice were inoculated by peroral (p.o.) or i.n. route with MCMV. In animals surviving a generalized infection, the virus could be demonstrated in salivary glands up to day 123 p.i. The virus was present in salivary glands, lungs and kidneys, but no virus was recovered from Gasserian ganglia (Svobodová *et al.*, 1986).

Spread of MHV-68 to the spleen was accompanied by a marked splenomegaly and the spleen cell number increased 2–3 times (Sunil-Chandra *et al.*, 1992a; Ehtisham *et al.*, 1993; Mistríková *et al.*, 1994). The spleen appeared to be a major site of MHV-68 persistence as the lymphocytes were found to be latently infected up to day 90 p.i. Similar results were obtained with MHV-72, the latency could be detected up to day 240 p.i. (Mistríková *et al.*, 1994). During primary infection, the atrophy of the thymus and spleen of clinically sick animals was observed. In contrast, in asymptomatic animals, a lymphoprolipherative response manifested by splenomegaly was relatively frequent (Sunil-Chandra *et al.*, 1992a). To determine the cell type harboring persisting virus, spleen cells from infected animals were separated into Ig-positive B cell-enriched, Ig-negative cell-enriched, and macrophage adhering to plastic- enriched fractions. These cells were co-cultivated with permissive BHK-21 cells in an infectious center assay. A consistent recovery and enrichment of infectious centers in the Ig-positive fraction clearly demonstrated that B lymphocytes are a major site of virus persistence/latency (Sunil-Chandra *et al.*, 1992b). After i.n. inoculation of MHV-72 to Balb/c mice the virus persisted in adherent lung mononuclear cells (AMCs). Infectious virus was occasionally detected during a period of 8 months p.i. lymphatic organs (thymus, spleen, and lymph nodes), bone marrow, alveolar and peritoneal AMCs, and lymphocytes and macrophages of peripheral blood by an indirect IF test and co-cultivation with permissive Vero cells. VN antibodies were detected during one-year period in the sera of infected mice, and their levels correlated with reactivation of latent MHV-72 (Mistríková *et al.*, 1994) .

Recently, a MHV-68 latency was demonstrated in two different hematopoetic cell types, F4/80-positive macrophages and CD19-positive B cells (Weck *et al.*, 1999a). B cells play a crucial role in maintenance of MHV-68 in latency and are important for controlling chronic MHV-68 infection (Weck *et al.*, 1999b). Infected cells can also be detected in lymph nodes and bone marrow (Nash *et al.*, 1996). Peritoneal macrophages were identified as further reservoir of latent MHV-72 (Mistríkova *et al.*, 1994) and MHV-68 (Weck *et al.*, 1999a). Mucosal epithelium can act as a non-lymphoid reservoir for gammaherpesvirus persistence and there is a two-way movement of virus between lymphoid and non-lymphoid compartments during persistence. Lung epithelial cells are a major site of gammaherpesvirus latency (Stewart *et al.*, 1998a).

A common feature of gammaherpesvirus infection is the genesis of lymphoproliferative disorders (LPDs). Inbred mice chronically infected with MHV-68 developed LPDs similar to those seen in patients infected with EBV. The frequency of LPDs over a period of 3 years was 9% of all infected animals; a half of them displayed high grade lymphomas associated with both lymphoid and non-lymphoid tissues (liver, lungs, and kidneys). In all cases of lymphomas studied so far, they had a mixed B cell phenotype. The B cells from the lymphomas were of clonal origin. A variable number of virus genome-positive cells were detected by *in situ* hybridization around the lymphomas (Sunil-Chandra *et al.*, 1994a).

Chronic infection of Balb/c mice with MHV-72 is associated with neoplasm development. A hundred Balb/c mice were infected with MHV-72 and observed for 2.5 years for tumors. Five mice developed tumors (one lymphoma, two non-differentiated lymphoblastomas, and two fibrosarcomas) and one mouse had lymphatic leukemia. The virus persisted frequently also in various organs of the neoplasm-bearing mice (Mistríková *et al.*, 1996b). Chronic infection of Balb/c mice with MHV-Šumava was associated with development of different types of neoplasm (Table 2).

Several cell lines were derived from MHV-68-infected mice with LPDs. A cell line (S11) of B lymphocyte origin was found to contain the MHV-68 genome. The cell line grew into tumors in nude mice. This is the first established MHV-68-positive B cell line which may become an invaluable tool for the study of MHV-68 in the future (Usherwood *et al.*, 1996b). Another cell line was derived from lungs of MHV-72-infected BALB/c mice with LPDs. This cell line stopped growing in its 35th passage due to the virus reactivation and cell destruction (J. Mistríková, unpublished data).

The blood picture was investigated in the group of a hundred MHV-72-infected mice during 2.5 years. Atypical lymphocytes in the acute phase of infection, the same as during the human EBV infection, were identified. In later

Table 2. Development of LPDs in mice experimentally infected with various isolates of MHV-68

Virus (isolate)	Mouse strain	No. of infected mice	Days p.i.	No. (%) of mice with LPDs
MHV-68 (Sunil-Chandra *et al.*, 1994)	Balb/c C57BL/10 CBA	220	165–834	25 (11%)
MHV-72 (Mistríková *et al.*, 1996)	Balb/c	100	240–870	13 (13%)
MHV-Šumava (J. Mistríková and M. Stančeková, unpublished data)	Balb-c	150	141–784	22 (14.6%)

LPDs = lymphoma-associated with lymphoid and non-lymphoid tissues (liver, lung, and kidney) and leukemia-like syndromes.

intervals of chronic phase of infection many hematological disorders were detected including several types of leukemia (Mistríková and Mrmusová, 1998). The atypical lymphocytes in the blood of MHV-72-infected nude mice were immunophenotyped as B cells (Rašlová *et al.*, 2000c).

In the first 2–3 weeks after MHV-68 infection the mice developed a marked splenomegaly with an increase in the number of B lymhocytes and of both $CD4^+$ and $CD8^+$ T lymphocytes. The number of latently infected cells in the spleen peaked at day 10 post i.p. infection and then declined to 10^6–10^7 cells per spleen. Depletion of $CD4^+$ T lymphocytes prevented the splenomegaly and greatly reduced the infective center level, while no effect on the long-term level of latently infected cells was found (Usherwood *et al.*, 1996c).

B cells are required for acute splenic infection but not for establishment of latency by MHV-68. The MHV-68 latency may be established in the absence of acute virus replication in B cells. In addition, MHV-68 can establish latency in a cell type other than mature B lymphocytes (Weck *et al.*, 1996).

B cell-deficient mice were infected to determine whether a latent infection with MHV-68 can be established in the mice lacking circulating B lymphocytes. The acute lung infection was not much changed but the virus clearance was slightly delayed in these mice. This indicates that antibodies to the virus are of little importance for the lung infection. Neither active (free) nor latent virus could be detected in the spleen in these mice. In addition, they did not develop splenomegaly (Usherwood *et al.*, 1996c).

MHV-68 has many similarities to EBV concerning infectious mononucleosis. The findings suggest that the infectious mononucleosis-like disease induced by MHV-68 is driven both by a cytokine provided by $CD4^+$ T cells and a viral "superantigen" presented by MHC class II glycoproteins to V beta 4^+ $CD8^+$ T cells (Tripp *et al.*, 1997).

MHV-68 causes severe large-vessel arteritis in mice lacking interferon (IFN) gamma responsiveness. IFN gamma is essential for control of chronic vascular pathology induced by MHV-68 and gammaherpesviruses are suggested as potential etiological agents of human vasculitis (Weck *et al.*, 1997).

MHV-68 has the ability to survive in acidic and proteolytic environment of the upper gastrointestinal tract and to productively infect intestinal epithelial cells (Peacock and Bost, 2000). These results are in accordance with those obtained *in vitro* (Stančeková *et al.*, 1992) The rapid development of systemic mononucleosis–like disease following intragastric administration of the virus (Peacock and Bost, 2000) suggests a normal route of infection for this pathogen in mice. Based on these data MHV-68 should be a candidate model for studying viral pathogenesis at a mucosal surface and exploring the possibility of cellular transformation of intestinal epithelial cells.As concluded by Simas and Efstathiou (1998) MHV-68 is a very useful model for the study of gammaherpesvirus pathogenesis in general.

Immunological characteristic

Little is known about the immunological response to gammaherpesviruses during primary infection. MHV-68 may be very useful in understanding of immunological characteristic of these viruses .

Mice inoculated i.n. with MHV-68 establish productive infection in the lungs, causing pneumonia involving alveolar epithelial cells and mononuclear cells surrounding airways and blood vesicles. The productive phase of infection lasts for about 10 days before clearance by the immune system. An intense inflammatory infiltration accompanies the primary infection and persists for 30 days p.i. (Sunil-Chandra *et al.*, 1992a).

The host response to MHV-68 infection in the lungs is characterized by infiltration of monocytes/macrophages but just a few polymorphonuclear leukocytes during the first 3 days of infection. By day 5 p.i., the number of macrophages

in broncho-alveolar lavage declines and the number of T cells increases. The latter cells are mostly of $CD8^+$ type; the $CD4^+$ type is rare. Surprisingly, no or very few B cells were detected in Balb/c mice during the primary infection (Nash and Sunil-Chandra, 1994; Nash *et al.*, 1996).

The appearance of a large number of $CD8^+$ T cells in the lungs suggests that these cells may play an important antiviral role in this tissue (Ehtisham *et al.*, 1993). Clearance of the primary infection in the lungs coincides with the peak of virus-specific $CD8^+$ T cells (Stevenson and Doherty, 1998). Depletion of $CD8^+$ T cells before infection leads to uncontrolled virus replication resulting in death (Ehtisam *et al.*, 1993). In the absence of $CD8^+$ T cells the infection progresses despite the presence of $CD4^+$ T cells and antibodies. The lack of $CD4^+$ T cells in MHC class II-deficient mice leads to a deficit in immune surveillance by $CD8^+$ T cells and the lack of antiviral antibodies (Cardin *et al.*, 1996).

MHV-68 multiplies in the respiratory epithelium after i.n. inoculation and then infects B cells in lymphoid germinal centers. A non-specific antigen B cell activation following MHV-68 infection is $CD4^+$-independent *in vitro*, but $CD4^+$-dependent *in vivo* (Stevenson and Doherty 1998). Interestingly, the cytotoxic activity is not mediated via perforin and is independent of $CD4^+$ T cells (Ehtisham *et al.*, 1993; Cardin *et al.*, 1996; Usherwood *et al.*, 1997).

The antibody response to MHV-68 infection develops slowly but then increases dramatically up to 2 weeks p.i. and remains at high levels thereafter (Stevenson and Doherty, 1998). The antibodies are apparently not essential for suppression of acute infection (Ehtisham *et al.*, 1993; Cardin *et al.*, 1996; Usherwood *et al.*, 1996a),

MHV-68 antibodies first appear at the end of the first week of i.n. infection (Kulkarni *et al.*, 1997). The same results were obtained with MHV-72 (Mistríková *et al.*, 1994). VN antibodies could be detected in the sera of MHV-72 infected mice 7–365 daysp.i. Their dynamics during one-year observation period shows two peaks, the first one occurring 17 days p.i. (titer of 512) and the second one occurring 3 months p.i. (titers of 512–1024) (Mistríková *et al.*, 1994).

The persistence of MHV-72 in lungs and peritoneal adherent mononuclear cells correlates with increased VN antibodies in mouse sera (Mistríková *et al.*, 1994). It seems that the maintenance of high levels of serum VN antibodies might play a role in limiting the virus spread during persistent infection.

Infection of mice induces pulmonary inflammatory response composed of T, B and NK cells and macrophages and stimulates activation and proliferation of T and B cells in the spleen (Kulkarni *et al.*, 1997). During splenomegaly the number of B as well as T cells doubles. A cytofluorometric analysis during MHV-68-induced splenomegaly revealed an increase in numbers of B lymphocytes and of both $CD4^+$ and $CD8^+$ T lymphocytes. A higher increase relative to uninfected spleens was in the $CD8^+$ population (Usherwood *et al.*, 1996c). The same results were obtained with MHV-72 and MHV-76 infections (M. Mrmusová, unpublished data). Consistent with the findings of Sunil-Chandra *et al.* (1992a) is the observation of marked splenomegaly in otherwise clinically asymptomatic MHV-72-infected mice (Mistríková *et al.*, 1994). It seems likely that the latently infected B cells in the spleen initiate splenomegaly because the infection of transgenic mice deficient in mature B cells is not associated with splenomegaly (Usherwood *et al.*, 1996a; Weck *et al.*, 1996). This underlines the importance of the infected B cell for the genesis of splenomegaly and the possible spread of the virus from lungs to spleen. MHV-68 induces proliferation of T lymphocytes early after infection. Splenic and peripheral blood activated T lymphocytes were found to continue dividing for at least a month after the initial virus challenge. These results are in accord with the idea that T cells are stimulated for a substantial time after the acute, lytic phase of virus infection (Hamilton-Easton *et al.*, 1999).

During acute infection, high levels IFN gamma and IL-6, low levels of IL-2 and IL-10, and undetectable levels of IL-4 and IL-5 were found (Sarawar *et al.*, 1996). Nevertheless, IFN gamma production is not essential for effective T cell cytotoxicity. Mice without a functional IFN gamma system are able to clear an acute viral infection (Sarawar *et al.*, 1997; Dutia *et al.*, 1997; Weck *et al.*, 1997). MHV-68 induces high levels of IL-6 in both native and primed lymphocyte populations. Mice homozygous for the deletion of IL-6 gene were used to investigate the role of this cytokine in MHV-68 infection. The results showed that IL-6 is not essential for clearance of the infection from lungs or for control of virus latency (Sarawar *et al.*, 1998).

MHV-68-induced infectious mononucleosis is characterized by transient lymphocytosis, an increase in the frequency of activated T cells ($CD8^+$, $CD4^+$, and $CD62^-$) in peripheral blood (Tripp *et al.*, 1997). Expansion of T cells is probably a response to infection of B cells. Indeed, a recent analysis of T cell receptor usage during MHV-68 infection revealed a disproportional rise in T cells in blood and spleen. The selective expansion of T cells indicates possible roles of the virus and the induced "superantigen" in the pathogenesis of an infectious mononucleosis-like disease (Tripp *et al.*, 1997).

A recombinat vaccinia virus expressing MHV-68 gp150 was used for immunization of mice. The vaccination resulted in the production of MHV-68 neutralizing antibodies. The immunized mice challenged i.n. with MHV-68 established latency but the virus-associated mononucleosis was abrogated. These results suggest that functional homologue of MHV-68 gp150, EBV gp350 may be effective as an immunogen to prevent EBV-associated infectious mononucleosis in humans which are EBV-seronegative (Stewart *et al.*, 1999). The peak level of latently infected spleen cells

was significantly reduced following vaccination that induced a response to the immunodominant $CD8^+$ T cell epitopes. However, this vaccination approach did not prevent the long-term establishment of latency or the development of the infectious mononucleosis-like syndrome in infected mice. The virus is able to establish latency efficiently despite strong immunological control of the lytic infection (Liu *et al.*, 1999).

Effect of immunosuppression on MHV-68 infection

Immunosuppressive effect of the antibiotic FK506 on MHV-72-infected Balb/c during acute and chronic of infection was investigated. In the immunosuppressed mice, the virus was detected in various organs and tissues at 2 times higher rate than in control mice. At later intervals of infection, the virus was still recovered in bone marrow, lymph nodes and peritoneal cells of immunosuppressed mice but not in control animals. In the chronically infected immunosuppressed mice, the virus was detected in lungs, thymus, bone marrow, spleen and peritoneal cells at 3.5 times higher rate than in control mice (Mistríková *et al.*, 1996a). In later intervals of chronic infection in immunosuppressed mice many hematological disorders were detected (Mistríková and Mrmusová, 1998; Mistríková *et al.*, 1999). The incidence of LPDs was greatly increased from 9% to 60% when MHV-68-infected mice were treated with cyclosporin A (Sunil-Chandra *et al.*, 1994a).

Effect of antiviral drugs on MHV-68 infection

Inhibition of MHV-68 infection was tested first with ACV (Sunil-Chandra *et al.*, 1993). In a persistent infection, linear virus DNA molecules were shown to be predominating, but low levels of circular virus DNA molecules were also present. Treatment of B cells with ACV resulted in a significant reduction of linear DNA content but had no effect on the circular DNA content. These data provided further evidence supporting not only earlier observations on B cells as a site of latency (Sunil-Chandra *et al.*, 1992b) but also suggest a possibility to use this *in vitro* model for the study of molecular basis of MHV-68 latency/persistence. ACV treatment of acutely MHV-68-infected mice markedly (about 10-fold) decreased the number of infectious centers in the spleen as compared to placebo-treated animals but was not effective once the MHV-68 latency was established. ACV minimized MHV-68 replication at the site of primary infection resulting in reduction of latently infected spleen lymphocytes (Sunil-Chandra *et al.*, 1994b). When the immunosuppression in Balb/c mice was used, ACV decreased virus titers in lungs but did not prolonge the survival time (Smee *et al.*, 1997). In comparison with the results from an earlier report, the ACV inhibitory effect was 1000-fold weaker than that in normal mice. ACV was shown to be less active than other inhibitors against MHV-68. The same is known for EBV. The inhibitory effect of 16 known inhibitors of EBV or other herpesviruses on MHV-68 infection *in vitro* was evaluated by Smee *et al.* (1997).

Another drug, gancyclovir (GCV) was shown to have *in vitro* a weaker antiviral effect than ACV; the effect of GCV was about 10% of that of nucleoside analogues (cyclic nucleoside phosphates). MHV-68- infected i.n. and cyclophosphamide-immunosuppressed mice were treated with ACV, fialuridin, cidofovir and placebo for 5 days starting 1 day after virus challenge. Cidofovir reduced the virus production in lungs over 3000-fold. Cidofovir was suggested as an excellent candidate for treating EBV infection in humans (Smee *et al.*, 1997). The lower *in vitro* susceptibility of the virus to ACV as compared to GCV was reported by Neyts and DeClercq (1998). Furthermore, cidofovir is very efficient in protecting mice against virus-induced death in the case of severe combined immunodeficiency (*in vivo)* as well as against MHV-68 replication in infected cells (*in vitro*). However, the susceptibility of MHV-68 and EBV to ACV, cidofovir, and adenovir but not to GCV, brivudin, and penciclovir suggests that MHV-68 is not always the optimal model for the study of antiviral strategies for EBV.

Recently, the ability of MHV-72 TK to phosphorylate several nucleoside analogues was also studied. MHV-72 TK was expressed via a mammalian expression vector in transfected TK-deficient cells and its substrate specificity for GCV, azidothymidine (AZT), brivudin and FUDR was evaluated. The latter was estimated as extremely toxic for MHV-72 TK-expressing cells at a 16-fold lower concentration than for cells expressing HVS-2 TK (Rašlová *et al.*, 2000b). In addition, a bystander effect was observed in mouse tumor fibroblastoid cells co-cultivated with MHV-72 TK-expressing cells in the presence of FUDR. Only 1% of MHV-72 TK-expressing cells were able to enhance the killing of mouse cells and to decrease their survival to 25%. These results demonstrate the efficiency of MHV-72 TK as a suicide gene provided FUDR is used. From the point of view of the currently intense search for novel and/or mutant TK genes facilitating a more effective killing of tumor cells at a less toxic concentration of drugs, MHV-72 TK gene appears to be useful in gene therapy.

Ecology and epidemiology

The natural role of MHV-68 infection in bank voles and wild mice involves close contact, probably respiratory transmission. In a study of dissemination of this virus, together 381 sera from murine rodents (*Apodemus flavicollis, Clethrionomys glareolus, Microtus arvalis,* and *Mus musculus*) trapped in different localities of Slovakia and Bohemia were examined for the presence of antibodies

to MHV-60, MHV-76 and MCMV-1. Their positivity for these antibodies varied from none to about 12% depending on the locality (Mistríková and Blaškovič, 1985).

Later, 935 sera from micromammals collected in 19 different localities of Slovakia and Bohemia were examined by a CF test for antibodies to MHV-68 and MCMV-1. Both viruses belonging to different subfamilies of the *Herpesviridae* family were found to occur in natural habitats in localities with various geographic, geological and climatic conditions at altitudes of 100–550 m above the sea level. The CF test used was not sufficiently specific for classification of these viruses into different *Herpesviridae* subfamilies (Blaškovič *et al.*, 1987a,b).

A total of 69 small mammals of 6 species were collected in localities of Marcelová and Kopáč (southwestern Slovakia) and used for a herpesvirus isolation. Two isolates, MHV-4556 and MHV-5682 were obtained. The both isolates killed suckling mice after i.c. or i.p. inoculation. Adult mice were killed 4–7 days after i.c. inoculation. Rabbit immune sera against MHV-72 and MHV-76 cross-reacted with MHV-4556 and MHV-5682 (Kožuch *et al.*, 1993).

Furthermore, the level of VN antibodies against MHV-68 in sera of different animal species sharing the biotop with small murine rodents was investigated. The antibodies were found in sera of red european dear (*Cervus elephus)*, fallow dear (*Dana dana)*, wild pigs (*Sus scrofa)* but not phasant (*Phasianus colchicus)* and muflon (*Ovis musimon*) (Mistríková *et al.*, 2000).

Recently it was found that laboratory workers exposed for relatively long time to MHV-68 developed a humoral response against this virus. Antibody titers ranging from 1:100 to 1:1000 were determined by enzyme-linked immunosorbent assay (ELISA) in sera of these persons. The MHV-68 antibody level correlated with the length of exposition to the immunogen. In addition, MHV-68 antibodies could be found also in sera of forest workers with possible contact to small murine rodents. Moreover, it was demonstrated that MHV-68 could infect human cells *in vitro* (Svobodová *et al.*, 1982a). Provided MHV-68 could overcome the species barrier also *in vivo* as indicated by specific humoral response in humans, this would indicate a pathogenic potential of MHV-68 also for humans. Although the pathogenicity of MHV-68 for humans was not yet proved it should be at least taken in consideration (Mistríková *et al.*, 2000).

Conclusions

Gammaherpesviruses have the ability to transform lymphocytes causing LPDs and malignancies in natural or experimental hosts. MHV-68 infection of laboratory mice presents an attractive animal model system for investigating the gammaherpesvirus pathogenesis including the host antiviral response. MHV-68 genome was cloned and completely sequenced and its ORFs, genes, primary transcripts and mature mRNAs are current foci of intense interest in searching for the relatedness of MHV-68 to other gammaherpesviruses.

The pattern of infection observed in mice infected with MHV-68 or MHV-72 is similar to that seen in EBV-infected man suffering from infectious mononucleosis. It is not yet known whether the mechanism of cell transformation observed in chronic MHV-68 infection is similar to that of EBV. Molecular and pathogenic similarities of MHV-68 to EBV identify this virus as useful model for the study of a human disease caused by EBV. The MHV-68 mouse model permits an *in vivo* study of such issues as the efficacy of vaccination and the tumorigenicity of virus-infected cells. An additional benefit of MHV-68 mouse model is the possibility of using the transgenic knockout technology for the study of host immune system components involved in antiviral defense. The selection of virus-specific proteins suitable as crucial components of a vaccine against gammaherpesviruses seems to be helpful for potential immunotherapeutic strategies in the future.

Characterization of a number of virus ORFs including those having their cellular counterparts offers an opportunity to assess their role in MHV-68 infection, latency and tumor induction. Recent description of MHV-68 genes affords the possibility to search for similar genes in other apparently phylogenetically much younger gammaherpesviruses.

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Note of the Editor-in-Chief. BoHV-4, HHV-8, OHV-1 and RCMV are not listed in the presently valid virus taxonomy (Murphy *et al.*, 1995).

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