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Vertical transmission of murine gammaherpesvirus 68 in mice

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Summary. – The mouse infected with murine gammaherpesvirus 68 (MHV-68) is accepted animal model for investigation of pathogenesis, oncogenesis, immunology and molecular biology of gammaherpesviruses in their natural host. However, little is known about the host range, epidemiology and pathogenesis of this natural pathogen of free-living murid rodents. Therefore we addressed the question of transplacental transmission of MHV-68 from pregnant Balb/c mice chronically infected with the virus to their fetuses and shedding of the virus by breast milk from chonically infected mothers to their offspring. The mothers were positive to infectious virus and viral antigen in various organs but mainly in the spleen and peritoneal *macrophages. Virus-neutralizing serum antibodies, and leukocyte count with a proportion of atypical lymphocytes were increasing to elevated values with the time of infection. The chronical infection resulted in premature termination of pregnancy or reduced number and size of newborns due to their retarded development. Out of 10 infected pregnant mice just one died and another developed a tumor. All these results confirm the vertical transmission of MHV-68 in mice, teratogenicity of the virus and the virus shedding by breast milk.

Keywords: murine gammaherpesvirus 68; mice, vertical transmission, teratogenicity, breast milk

Introduction

Herpesviruses are known for their ability to establish a lifelong latent infection. MHV-68 establishes a latent infection in B-lymphocytes following an acute respiratory infection of mice (Sunil-Chandra *et al*., 1994). At late stages of latent MHV-68 infection, 10% of mice develop a lymphoproliferative disease with a high proportion (~50%) of high-grade lymphomas (Sunil-Chandra *et al*., 1994; Mistríková *et al*., 1996). Viral DNA was detected in alveolar epithelial cells of lungs, macrophages, trafficking B-lymphocytes carrying the genome to the spleen and lymph nodes, NK cells and dendritic cells (Stewart *et al*., 1998).

The mechanism by which B-cells, mononuclear leukocytes and dendritic cells become latent carriers of MHV-68 DNA is still obscure. The importance of MHV-68 grows due its worldwide use as an experimental model for studying a gammaherpesvirus infection. MHV-68 has been classified to the *Murid herpesvirus 4* species, the *Rhadinovirus* genus, the *Gammaherpesvirinae* subfamily (Fauquet *et al*., 2005). Epstein-Barr virus (EBV), an important human pathogen is an oncogenic gammaherpesvirus. It is etiological agent of Burkitt`s lymphoma, nasopharyngeal carcinoma and Hodgkin`s disease. However, the study of EBV is limited by its strict host range.

MHV-68 is a long-time object of our research in gammaherpesviruses. Being isolated from a bank vole (*Myodes glareolus*) in Slovakia, it is a natural pathogen of free-living murid rodents (Blaškovič *et al*. 1980). Neutralizing serum MHV-68 antibodies were found in animals of other species living with infected rodents in the same biotop such as *A. flavicollis, C. glareolus, M. arvalis* (Mistríková and Blaškovič, 1985), fallow dear, wild boars, deer, sheep, foxes and muflons as well as in humans, namely laboratory personnel working with this virus (Marková *et al*., 2007; Hamzová *et al*., 2005; Mistríková *et al*., 2000), hunters (Marková *et al*., 2007) and general population

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Abbreviations: EBV = Epstein-Barr virus; IF = immunofluorescence; i.n. = intranasal(ly); MHV-60, MHV-68, MHV-72, MHV-76, MHV-78, and MHV-Šumava = murine gammaherpesviruses 60, 68, 72, 76, 78, and Šumava, respectively; p.i. = post infection

(Mistríková *et al.*, 2000; Hricová and Mistríková, 2008). In studying the incidence of MHV-68 infection in free living rodents in UK, Blasdel *et al.* (2003) found its endemicity in *A. flavicollis* but not in *C. glareolus* and *M. agrestis*. A long-term study of MHV-68 antibodies in a mixed population of *C.glareolus* and *A. sylvaticus* in UK revealed their higher prevalence in the latter host, thereby supporting a previous view that *A. sylvaticus* is the major natural host of MHV-68, even though it has been originally isolated from *M. glareolus* (Telfer *et al.*, 2007). An investigation of incidence of 13 different viruses in *M. domesticus* in UK showed for MHV-68 values of 3–13% (Becker *et al.*, 2007). All these data have led to a conclusion that MHV-68 infects in the nature various rodent species belonging to diverse, phylogenetically distant families, and to a hypothesis, that it could be transmitted from infected rodents vertically and horizontally to other animals living in common biotops. The virus was detected in breast milk, urine, saliva and tear glands at day 14 post infection (p.i.)(Hricová and Mistríková, 2008). Out of intranasal, peroral, intramuscular, intraperitoneal and subcutaneous routes of infection the first two proved most effective.

In spite of the fact that the transmission of MHV-68 within murine rodents and to other animals and humans has been basically proved, its precise ways were not yet clarified. Therefore this study was aimed at the vertical transmission of MHV-68 as one of possible ways of virus shedding.

Materials and Methods

Virus. A MHV-68 stock was prepared in Vero cells. At 72–96 hrs p.i., the cells were harvested, sonicated, clarified by low-speed centrigugation, and stored at -70°C until use.

Cells. Vero cells were cultivated in DMEM supplemented with 7% of FCS, 300 μg/ml glutamine, and 80 μg/ml gentamicin.

Mice. BALB/c mice were supplied by the Faculty of Veterinary Medicine, Brno, Czech Republic. They were kept under standard housing conditions.

Experiments on mice. A group (15) of 6 week-old female BALB/c mice were infected intranasally (i.n.) with 2 x 10^4 PFU (20 μl) of the virus per mouse under light anesthesia with ether (Fig. 1). After 4–14 months, they were mated up with healthy adult males, and following 21 days of gravidity, they brought for a litter. Both the mothers and newborns were sacrificed by cervical dislocation under ether anesthesia and examined for infectious virus and viral particle antigen. From the mothers, the blood, thymus, lungs, cord, liver, spleen, bone marrow, peritoneal macrophages, lymph nodes, kidneys, mammary glands, brain, and tumors were removed, homogenized and stored at -70°C until assayed. Blood samples were taken from *sinus orbitalis* of mothers before sacrificing.

Blood cell and differential leukocyte counts were done in standard manner.

Cytological examination of infected coverslip cell cultures was done following fixation and hematoxylin-eosin staining.

Infectious virus was assayed by plaque titration in Vero cells in standard manner. CPE was read after incubation for 3–7 days at 37°C in 5% CO_2 and hematoxylin-eosin staining.

Immunofluorescence (IF) assay of virus particle antigen was done using an indirect procedure. Cells in suspension (100 μl) were incubated with a polyclonal rabbit MHV-68 antibody (dilution 1:100) at 37°C for 45 mins, stained with FITC-conjugated goat anti-rabbit IgG (H + L) (1:1000 dilution, Immunotech, Slovak Republic) at 37° C for 45 mins, mounted, and examined under fluorescence microscope. Between individual steps a standard washing with PBS was done. The percentage of cells positive for fluorescence was calculated.

Results and Discussion

In the experiment on mice, females were infected with MHV-68 and 4–14 months later they were mated up with non-

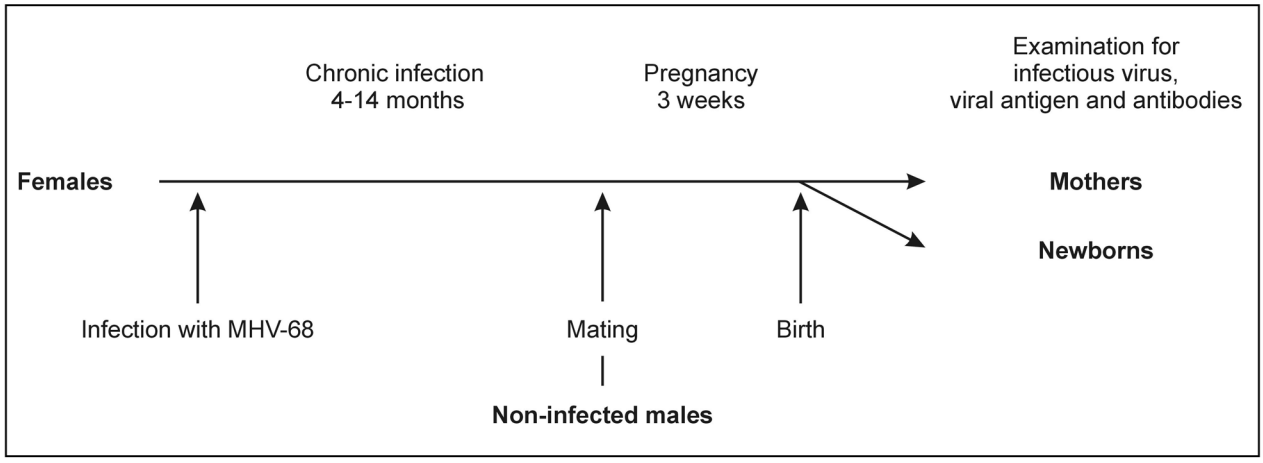

Fig. 1

Table 1. Transmission of MHV-68 from chronically infected pregnant mice to newborns

Time between infection and mating (months)	Number of newborns	Infectious virus in newborns[1]	Antibody titer in the serum of infected mothers	Infectious virus in infected mothers[2,3]	Presence of tumors in infeceted females / lethality of chronic infection
4	2	+	64	+	-/-
8	3	+	4	+	-/-
9	4	+	1024	+	-/-
9	4	+	128	++	+/-
10	3	+	8	+	–
10	2	+	16	+++	+/-
12	3	+	64	+	-/-
12	3	+	128	+	-/-
12	3	+	64–128	+	-/-
14	2	+	64	+	-/-

[1](+) = positive, (–) = negative. [2]Positivity only in undiluted sample (+) or also in its 10^{-1} (++) or 10^{-2} dilution (+++). [3]Results deduced from Table 2.

infected males. After 3 weeks of pregnancy, they brought for a litter (Fig. 1). The infected females before and after mating, and the infected mothers as well as newborns after the birth were observed and examined for various characteritsics of health status, virus infection, and humoral immune response and birth.

The i.n. infection of female mice with MHV-68 resulted in chronic infection with acute (0–1 month p.i.) and chronic (2–14 months p.i.) phases as reported previously (Sunil-Chandra *et al.*, 1992, 1993, 1994; Mistríková *et al.*, 1996; Stewart *et al.*, 1998). The chronic infection is characteristic by virus latency with presence of episomal viral DNA, expression of latency genes and spontaneous or induced reactivation. The reactivated virus, detectable by infectivity and antigenicity, is considered responsible for induction of virus-neutralizing antibodies and oncogenicity. However, a potential role of latent virus in viral oncogenicity still cannot be excluded.

The observation of female mice after infection, during pregnancy and after giving birth to newborns did not reveal any apparent symptoms of illness during either acute or chronic phase of infection except one death at 10 months p.i. and a tumor in one mouse at 9 months p.i. The infection caused a retardation of fetus development and premature termination of pregnancy, both resulting in a reduced number (3–4 vs. 9–11) and size of newborns. All the infected mothers exhibited infectious virus as well as serum virus-neutralizing antibodies no matter the chronic infection

Table 2. Presence of infectious virus and viral particle antigen in various organs or tissues of mothers chronically infected with MHV-68

Organ/tissue	Time p.i. (months)					
	4	8	9	10	12	14
Blood	+	+	+	+	+	+
Thymus	–	+	–	–	+	–
Lungs	+	+	+	+	+	+
Cord	–	–	–	–	–	–
Liver	+	++	+	+	+	+
Spleen[1]	++	++	++	++	++	++
Bone marrow	–	+	–	+	–	–
Peritoneal macrophages[1]	++	++	+	+	++	+
Lymph nodes	+	+	–	–	+	+
Kidneys	++	++	+	+	–	–
Mammary glands	+	+	+	+	+	+
Milk	+	+	+	+	+	+
Brain	+	–	–	+	–	–
Tumor	–	–	–	+	–	–

(+) = positive, (–) = negative. Positivity only in undiluted sample (+) or also in its 10^{-1} dilution (++). [1]Positivity for both infectious virus and viral particle antigen is underlined (+ , ++).

Table 3. Leukocyte count and proportion of atypical lymphocytes in mothers chronically infected with MHV-68

	Time between infection and mating (months)					
Leukocyte count	4	8	9	10	12	14
	6400	8000	8600	11000	9000	14000
Proportion (%) of atypical lymphocytes	4	7	9	7	10	15

before mating lasted 4 or 14 months. Rather low antibody titers reached a maximum of 1024 at 9 months p.i. (Table 1). The finding of virus positivity of all newborns born from chronically infected mice represents a direct proof of vertical transmission of MHV-68 in mice, i.e. a transplacental transmission of the virus from pregnant females to fetuses and consequently to newborns.

Examination of various organs or tissues of infected mothers for infectious virus and viral particle antigen revealed their rather low levels in the blood, thymus, lungs, cord, liver, spleen, bone marrow, peritoneal macrophages, lymph nodes, kidneys, mammary glands, and brain. The virus presence was most marked in the spleen and peritoneal macrophages. Surprisingly, the virus was also detected in a lymphoma in one mouse at 10 months p.i. (Table 2).

The examination of the blood of infected females at 4 months p.i. revealed a normal leukocyte count (6400) but a reduced proportion of atypical lymphocytes (4%), both values progressively increasing with the duration of infection and reaching maximum values after 14 months of infection (14,000 and 15%, respectively (Table 3). The observed atypical lymphocytes could be classified as lymphoblasts (developing stages of neutrofile polymorphonuclear leukocytes). At last, infectious virus was found also in breast milk of infected mothers, thus proving the possibility of virus shedding by breast milk to newborns (data not shown).

These results confirmed the presence of infectious virus in chronically infected mice, evidently caused by reactivation of latent virus. It is assumed that it is the reactivated virus which is subject to transplacental transmission and shedding by breast milk. At the same time, these results also demonstrated the viral teratogenicity resulting in a retarded fetus development, premature birth, and reduced number and size of newborns.

The ability of MHV-68 to induce a chronic infection was proven for the first time by explantation of the lungs, spleen and kidneys from mice surviving for several months p.i. (Rajčáni *et al.*, 1985). The persistence of MHV-68 in B-lymphocytes was characterized as a non-productive latency (Sunil-Chandra *et al.*, 1993). In this work, we found infectious virus and virus particle antigen in many organs or tisues of chronically infected mothers. It can be assumed that the primary site of virus multiplication in acute phase of infection is in lungs with the virus spreading to the lymphatic system by hematogenous route. In chronic phase of infection, we detected most of the virus in the spleen and peritoneal macrophages. The persistence of the virus in many organs confirms its affinity not only to cells of lymphatic system but also to neural (brain) and glandular (mammary glands) cells. Our finding of the virus presence in all tested organs or tissues of infected mice throughout entire chronic phase of infection corresponds to a similar presence of virus-neutralizing antibodies in the serum, though in low titers, from 2 to 27 months p.i. (Table 1, data not shown).

Our findings indicate also the possibility of secretion of the virus by breast milk similarly to EBV (Junker *et al.*, 1991), which is known to induce infectious mononucleosis, a benign self-limited lymphoproliferative disease characterized by development of lymphoid cells into atypical lymphocytes (Tomkinson *et al.*, 1987). We have observed atypical lymphocytes in the blood first with murine gammaherpesvirus 72 (MHV-72) infecting immunocompetent mice (Mistríková and Mrmusová, 1998) and T-cell-deficient nude mice (Rašlová *et al.*, 2000). The atypical leukocytes observed with MHV-72 or MHV-68 resembled those found in the patients with myeloid leukemia or infectious mononucleosis (Mistríková and Mrmusová, 1998). In comparison with other murine gammaherpesviruses, MHV-68 caused very low levels of atypical lymphocytes, a symptom usual in infectious mononucleosis or leukemia syndrome (Mistríková *et al.*, 2004).

Our finding of a tumor in one mouse at 9 months p.i. with MHV-68 corresponds to previous data (Sunil-Chandra *et al.*, 1994). As for the oncogenicity of murine gammaherpesviruses in general, it shows a wide range, from none (MHV-76) to 7% (MHV-78), 11% (MHV-68), 13% (MHV-72), 15% (MHV-Šumava), and 22% (MHV-60) (Chalupková *et al.*, 2008; Mrmusová *et al.*, 2003; Sunil-Chandra *et al.*, 1994; Mistríková *et al.*, 1996, 2002; Pappová *et al.*, 2004). It is assumed that the diverse oncogenicity of individual murine gammaherpesviruses reflects their different genome structure. For example, the non-oncogenic MHV-76 lacks the entire 9.5 kbp 5′-end of genome containing M1-M4 genes and eight tRNA-like sequences as compared with MHV-68 (Clambey *et al.*, 2002).

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