REPLICATION AND PERSISTENCE OF MURINE HERPESVIRUS 72 IN LYMPHATIC SYSTEM AND PERIPHERAL BLOOD MONONUCLEAR CELLS OF BALB/C MICE

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Summary. – After intranasal inoculation of murine herpesvirus 72 (MHV-72) to Balb/c mice the virus persisted in adherent lung mononuclear cells (AMC). Infectious virus was occasionally detected during a period of eight months after infection in lymphatic organs (thymus, spleen, lymph nodes), bone marrow, alveolar and peritoneal AMC, and lymphocytes and macrophages of peripheral blood by indirect immunofluorscence and cocultivation with permissive VERO cells. Mouse B-cell lines NS0 and SP2/0 were permissive for MHV-72 infection when inoculated *in vitro*. Virus-neutralizing antibodies have been detected during one year period in the sera of infected mice, and their levels correlated with activation of the latent MHV-72.

Key words: murine herpesvirus; gammaherpesviruses; pathogenesis; lymphatic system; latent infection

Introduction

From 2 species of small free-living rodents (*Apodemus flavicollis* and *Clethrionomys glareolus*), 5 viral strains (MHV-60, 68, 72, 76 and 78), were isolated (Blaškovič *et al.*, 1980). The virus is widespread in the population of free-living small rodents. Serological examination demonstrated 0 – 12% MHV-antibody prevalence, depending on the locality and the season when the mice had been trapped (Mistríková, 1985; Blaškovič *et al.*, 1987).

On the basis of their antigenic relatedness it was confirmed that the rodent herpesvirus isolates crossreacted antigenically and were serologically distinct from murine CMV (Svobodová *et al.*, 1982*b*).

The study of pathogenesis of MHV strains demonstrated that they persisted in lungs (Rajčáni *et al.*, 1985) and unlike to murine CMV they were never isolated from salivary glands of experimentally infected mice (Svobodová *et al.*, 1983). Unlike to HSV type 1, MHV spread to different organs by haematogenic route and not along nerves, in spite of the fact that it could be detected in Gasserian ganglia (Rajčáni *et al.*, 1987).

In 1990 partial nucleotide sequence analysis was performed by assignment of seven EBV DNA segments to the restriction fragments of MHV-68 DNA in order to determine the genome organization and its relatedness to that of viruses from the gammaherpesvirus group (Efstathiou *et al.*, 1990*a*, 1990*b*). Although regarding their biological properties MHV was originally included into the subgroup of *Alphaherpesvirinae* (Svobodová *et al.*, 1982*b*), the genomic analysis showed its relatedness to the subgroup *Gammaherpesvirinae*.

MHV may be a suitable model for studying the pathogenesis of gammaherpesviruses helping to explain the mechanism of multiplication of human lymphotropic gammaherpesviruses. Chandra *et al.* (1992*a*) showed that spleen was the major site of the persistence of the strain MHV-68 when detecting latently infected cells for up to 90 days p.i. This observation was confirmed on B- and T-cell lines *in vitro* (Chandra *et al.*, 1993).

The aim of the present work was to investigate the role of the cells of lymphatic system infected with MHV-72 strain during one year period after p.i. The main attention was focused on the role of AMC in virus persistence.

Materials and Methods

Viruses and cells. The stock of MHV-72 was prepared by infection of African green monkey kidney cells (VERO) kept at 37 °C in Eagle's Bassal medium (BEM) supplemented with 7% of inactivated bovine serum, glutamine (3 g/1000 ml) and antibiotics (100 units of penicillin and 100 μg of streptomycine per ml). Cell suspension was clarified by centrifugation, the supernatant was dispensed into 1 ml aliquots and stored at -70 °C until used. The titer of the stock virus was approximately 10^7 $TCID_{50}$/ml. SP2/0-B and NS0-B myeloma cell lines were originally derived from MOPC 21 myeloma of BALB/c mice (Kohler and Milstein, 1975, 1976). L-12-10 B- or T- cells were kindly supplied by Dr. Balážová from the Institute of Experimental Oncology of the Slovak Academy of Sciences in Bratislava. CEM-ss-human T-lymphocytes (Nara and Fischinger, 1988). U-937 macrophages, a human hematopoietic (histiocytic) cell line was described by Sundstrom and Nilson (1976). The cells were grown in RPMI-1640 medium containing 10% of foetal calf serum (FCS) and glutamine (3 g/1000 ml) and cultivated at 37 °C in 5% CO_2 for 3 days at concentration 3×10^4 cells/ml.

Mice. Newborn or 21 day-old female BALB/c mice were obtained from the Institute of Virology of the Slovak Academy of Sciences. Groups of 14 and 21 day-old female mice were inoculated with 0.02 ml doses containing 2×10^4 $TCID_{50}$ MHV-72 per mice. Groups of newborn mice were inoculated perorally with approximatelly 0.01 ml per mice.

Detection of antibodies and infectious virus in mouse sera and organs. Blood samples were taken from *sinus orbitalis* at different times p.i. The obtained sera were inactivated at 56 °C for 30 mins and stored at -20 °C until examined by virus neutralization test. Portions of blood samples were mixed with a drop of heparin diluted 1:100 (final heparin concentration 2 – 4μg/ml). Peripheral blood leukocytes were obtained by Ficoll-Verografin gradient centrifugation and purification by centrifugation in PBS pH 7.2. For peritoneal macrophages isolation each mouse peritoneal cavity was perfused with 20 ml PBS. 10% suspensions of thymus, spleen, lymph nodes and bone marrow were prepared in BEM supplemented with 5% of heat inactivated calf or bovine serum and antibiotics by homogenization and filtration through a cotton wool. The obtained cells were washed 3 times with RPMI-1640 medium supplemented with 5% heat-inactivated calf serum and stored at -70 °C until used. The erythrocytes present in spleen suspensions were lysed with 0.85% NH_4Cl. The remaining splenocytes were washed 3 times with RPMI-1640 medium and stored at -70 °C until used. Macrophages from lungs or peritoneal exudates were prepared by perfusion of alveols or peritoneal cavities with PBS. The obtained cells were pelleted by centrifugation, washed with RPMI-1640 medium supplemented with 5% or 10% heat-inactivated FCS and stored at -70 °C until used. Peripheral blood AMC cells were separated from the suspension of leukocytes by a 40 mins adsorption to a plastic surface. The adherent cells were separated and stored until use. Five ml of the cell suspensions with 10^7 cells were plated into 5 cm plastic Petri dishes. The cells were kept for 40 mins at 37 °C in CO_2 incubator with 5% CO_2 atmosfere. After carefull removal of non-adherent cells, the adherent cells were then recovered by a vigorous washing from the surface into RPMI-1640 medium with 10% FCS (Wood, 1979). Infectious virus titers of cellular extracts expressed in $TCID_{50}$ were determined in VERO cells grown in the same medium. The CPE was read 6 to 9 days after p.i.

Indirect immunofluorescence. The presence of the virus antigen was determined by indirect immunofluorescence and the percentage of positive cells was calculated. Suspensions of 5×10^3 cells in 0.01 ml obtained from lymphatic tissue of the infected mice were fixed on cover glasses with acetone or methanol and stained with rabbit anti-MHV-72 immune serum diluted 1:4 (titer 1:256) and with FITC-conjugated Sw-A-R/Ig (obtained from the Institute of Sera and Vaccines, Prague).

Preparation of hyperimmune serum. MHV-72 was propagated in VERO cells and cell-associated virus was obtained by sonication of cell suspensions and their clarification by centrifugation. The virus was further purified by two cycles of differential centrifugation. The second centrifugation was performed in a Ficoll gradient. The final step was virus sedimentation on a sucrose cushion. The purified virus in Freund's complete adjuvant was inoculated intramusculaly into rabbits. Booster injections in Feund's complete adjuvant were given at 4 weeks intervals. The sera obtained after animal bleeding were tested for the presence of virus neutralization antibodies.

Virus neutralization test. The test was performed with different dilutions of hyperimmune sera (from rabbits and mice) to which 100 $TCID_{50}$ of the appropriate virus was added. The virus-serum mixtures were incubated at 37 °C for 90 mins and then inoculated into 24 – 48 hrs-old VERO cell cultures according to the growth requirements of the respective virus. The titer was calculated after 5 – 6 days when a complete CPE was observed.

Preparation of cell cultures for cytological examination. Infected as well as uninfected cell cultures grown on glass cover slips were removed from the culture tubes at different time intervals. They were fixed in Carnoy's solution for 15 – 60 mins and then transferred to 96% alcohol for one or more days. The fixed specimens were stained with haematoxylin-eosin.

Results

MHV-72 was frequently lethal for new-borne mice which died 5 – 9 days after peroral infection; in contrast, 14 and 21 day-old mice did not show apparent symptoms of illness. The presence of the virus was followed in the lymphatic system of 14 day-old mice infected intranasally with 2×10^5 $TCID_{50}$ of virus. Even if the virus was given in much higher doses to 14 or 21 day-old mice, they did not show apparent symptoms of illness. The infected mice were sacrificed between 2 days to 8 months after infection when samples of blood, thymus, spleen, lymph nodes and bone marrow were taken. Alveolar and peritoneal AMC were obtained by perfusion. Lymphocytes and macrophages were obtained from lymphatic organs, perfusion fluid and periferal blood, and the presence of MHV-72 and its antigens were investigated. Results of these experiments are demonstrated in Table 1, Figs. 1 and 2. The presence of

Table 1. Assay of infectious virus and immunofluorescence in lymphatic cells of mice infected with MHV-72

Cells tissue	Assay	2 days	7 days	14 days	28 days	3 months	8 months
Peripheral blood macrophages	$TCID_{50}$/ml	0	<10	10^2	$10^{2.5}$	0	0
	IF	–	±	+	+	–	–
Peripheral blood lymphocytes	$TCID_{50}$/ml	0	0	<10	$10^{1.5}$	0	0
	IF	–	±	+	–	±	±
Thymocytes	$TCID_{50}$/ml	<10	10^2	$10^{2.5}$	10^2	<10	0
	IF	±	+	+	+	+	±
Splenocytes	$TCID_{50}$/ml	<10	10^3	10^2	10^2	<10	<10
	IF	±	+	+	+	+	+
Bone marrow	$TCID_{50}$/ml	0	$10^{1.5}$	<10	<10	<10	0
	IF	–	±	–	–	±	–
Lymph nodes	$TCID_{50}$/ml	0	10^2	<10	$10^{1.5}$	<10	0
	IF	–	+	–	–	±	+
Peritoneal macrophages	$TCID_{50}$/ml	<10	10^2	<10	$10^{2.5}$	10^2	<10
	IF	±	+	+	±	+	+

Mice were infected intranasally with 2 x 10^5 $TCID_{50}$ in 0.01 ml.
IF – immunofluorescence evaluated as (-) for all cells negative; (±), (+) and (++) for 1%, 2 – 4% and 4 – 10% of all cells positive.
0 – virus detected neither in the 1st nor in the 2nd cell culture subpassage.
<10 – virus detected in the 1st cell culture subpassage.

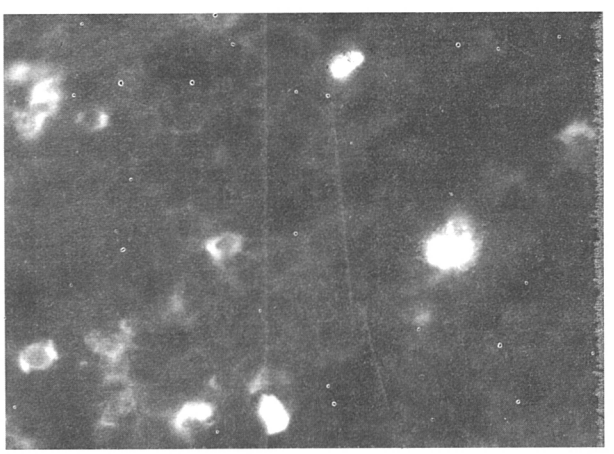

Fig. 1
Focal positive immunofluorescence of MHV-72 antigen in thymus of mice 14 days p.i. (magnification 1625 x)

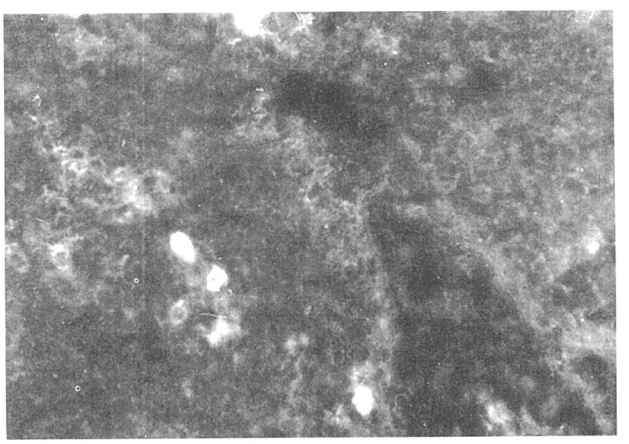

Fig. 2
Positive immunofluorescence of MHV-72 antigen in splenocytes of spleen trabecules of mice 14 days p.i. (magnification 1625 x)

infectious virus and virus antigen was detected in the investigated organs and cells of lymphatic system from day 7 p.i. The highest virus titer was present on 7 day in splenocytes, while the largest amount of the virus persisted in peritoneal macrophages for 3 months.

The first series of *in vivo* experiments confirmed the affinity of MHV-72 to cells of lymphatic system. T- and B-lymphocytes or macrophages were separated from peripheral blood of infected mice. Since common rosette tests using human blood were not successful, it was decided to

Table 2. Assay of virus antigen by immunofluorescence in various lymphatic cell lines infected with MHV-72

Cell line	Passage No.						
	1	2	3	4	5	6	7
NS0 -B-lymphocytes	+	+	+	+	+	±	–
SP2/0 -B-lymfphocytes	+	+	+	+	±	+	+
L-12-10 lymphocytes	±	±	±	–	–	±	–
U-937-macrophages	–	–	–	–	–	–	–
CEM-ss -T-lymphocytes	–	–	–	–	–	–	–

For legend see Table 1.

try some stable lymphoblastoid cell lines. Mouse lymphoblastoid cell lines NS0 and SP2/0, T lymphoblastoid cell line CEM-ss, human macrophage cell line U-937 and mouse lymphoblastoid cell line L-12-10 with unknown T- or B-lineage were used.

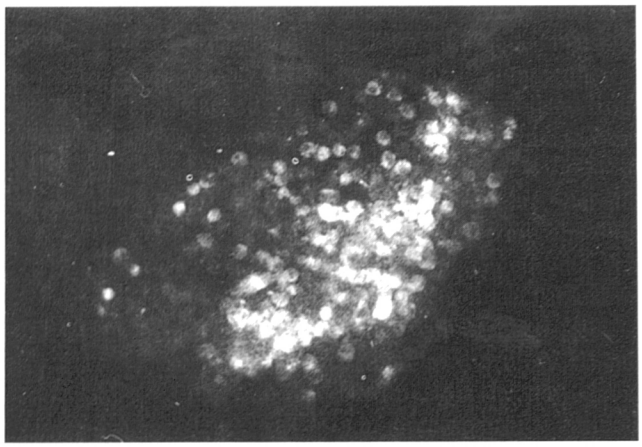

Fig. 3
Positive immunofluorescence of MHV-72 antigen in NS0 cells in culture/in the 3rd passage p.i. (magnification 1625 x)

Table 3. Presence of MHV-72 in alveolar AMC of mice at different stages of infection

	Days p.i.														
Assay	2	7	10	14	17	21	28	35	42	49	75	90	120	300	365
$TCID_{50}$/ml	10^2	10^3	10^4	$10^{3.5}$	10^3	10^5	10^2	10^2	10^3	10^2	10	10	10	0	ND
	+	+	+	+	+	+	±	–	+	+	–	–	–	–	–

For legend see Table 1.
ND – not done.

Table 4. Presence of MHV-72 in peritoneal AMC of mice at different stages of infection

	Days p.i.														
Assay	2	7	10	14	17	21	28	35	42	49	75	90	120	300	365
$TCID_{50}$/ml	0	0	0	0	10	10^4	ND	ND	10^5	10	10^4	10^5	ND	10^2	ND
	–	–	–	–	–	+	±	–	+	±	+	+	+	–	–

For legend see Table 1.
ND – not done.

Table 5. Infection of peritoneal AMC with MHV-72 in vitro

	Days p.i.		
	7	10	18
Extracellular virus ($TCID_{50}$/ml)	10^3	103	10^2
Intracellular virus ($TCID_{50}$/ml)	10^3	103	10^2
IF	+++	+	+

For legend see Table 1.

The results obtained with infection of the above mentioned cell lines are shown in Table 2 and Fig. 3. Intracellular and extracelular virus could be demonstrated in cells in which virus antigen was detected by cultivation with VERO cells. The results obtained with lymphoblast cell lines proved the B-lymphotropism of MHV-72.

Since the experiments *in vivo* demonstrated that the virus persists in peritoneal adherent cells for a long time, our attention was then focused on separation of macrophages from mice at different stages of infection. These macrophages were further cultured *in vitro* in order to test the virus release into cultivation medium.

The results obtained with lung alveolar AMC (Table 3) show that they were infectious 2 – 120 days p.i. of mice. The results obtained with peritoneal AMC (Table 4) show infectious virus could be recovered from them 17 – 120 days p.i.

In another experiment, peritoneal adherent cells obtained from individual samples at different intervals p.i. were cultured *in vitro* (Table 6). The virus antigen was detected

Table 6. Cultivation of peritoneal AMC obtained from mice at different stages of infection with MHV-72

Time after infection of mice (days)	Duration of cultivation of perit.macrophages (days)	Extracellular virus ($TCID_{50}$)	Intracellular virus ($TCID_{50}$)	IF
2	12	0	0	–
	15	0	0	–
7	10	0	0	–
	15	0	0	–
	20	0	0	–
	24	0	0	–
10	10	0	0	–
	15	0	0	–
	20	0	0	–
17	5	0	0	±
	10	<10	10	±
28	5	0	0	+
	10	10	10	++
35	5	0	0	+
	10	0	0	+
42	10	0	0	+
	15	10	10	±
49	15	10	10	+
75	10	0	0	+
	15	10	10	+
90	10	10	10	+
300	10	0	10	ND
	15	0	0	+
	20	0	0	ND

For legend see Table 1.
ND – not done.

of 10 $TCID_{50}$ per cell. Immunofluorescent antigen was seen in up to 50% of infected cells (Table 5). The intra- and extracellular virus titers show that these cells are permissive for MHV-72.

The persistence of MHV-72 in the lung and peritoneal AMC was associated with increased virus-neutralization antibody titers in sera of mice. The virus-neutralization antibodies could be also detected in the sera of infected mice 2 – 365 day p.i. Their dynamics during one year observation period shows two peaks, the first one occuring 17 days p.i. (titer 1:512), and the second one occuring 3 months p.i. (titer 1:512 – 1:1024).

Discussion

We found MHV-72 and its antigen in thymus, spleen, bone marrow, lymph nodes, lung and peritoneal AMC, peripheral blood AMC and blood lymphocytes. The primary site of viral multiplication were lung AMC from which the virus spread to other organs or cells of lymphatic system by haematogenous route.

Consistent with the findings of other authors (Chandra *et al.*, 1992*a*) is our observation of a marked splenomegaly in otherwise clinically asymptomatic mice. On the other hand, atrophy of spleen or thymus was not observed at any stage of infection.

Pathogenetic studies confirmed that the virus was present mainly in B-lymphocytes but probably also in T cells since the virus was detected in thymus shortly after infection. MHV-72 replicates in mouse B-lymphoblastoid cell lines NS0, SP2/0 but neither in human T cell line CEM-ss nor in human macrophage cell line U-937. Similar observations were published by other authors (Chandra *et al.*, 1992*b*). With regard to these findings it can be concluded that cells of

Table 7. Virus-neutralization antibodies in serum of mice infected with MHV-72

Days p.i.	7	10	14	17	28	35	42	49	75	90	120	300	365
Titer of antibodies	4	8	32/64	512	256	128	128/256	64/128	128	512/1024	256/512	128/256	128/256

in cultured cells originating from Balb/c mice 17 – 300 days p.i. Intra- and extracelular virus was recovered mostly on days 10 – 15 in cultures from cells obtained at the same intervals p.i. These results show that peritoneal adherent cells carried the virus for a long time period and that it was released into medium within 10 – 15 days in culture.

To prove that peritoneal macrophages are sensitive to MHV-72 infection *in vitro*, these cells originating from healthy adult mice were infected with virus at multiplicity

the lymphatic system, mainly B-lymphocytes, are permissive for MHV-72 under conditions both *in vivo* and *in vitro*.

Particular studies of peripheral blood AMC infected with MHV-72 *in vivo* and *in vitro* confirmed their important role for dissemination and latency of the virus in organism. It was found that the peripheral AMC from non-infected mice were sensitive to MHV-72 infection and the virus or its antigen was detected 7 – 18 days p.i. in 4 – 50 % of the cells (Table 5.). Cultured peritoneal AMC monolayers obtained

from Balb/c mice at different intervals p.i. (Table 6.) showed that the virus or its antigen was detected only at later stages of infection, beginning on days 17 – 21 p.i. and the virus was still recoverable from these cells 300 days p.i. Reactivated virus was released from cells into cultivation medium at later intervals , i.e. by 10 – 15 days in culture . It seems that the AMC play an important role in latency and reactivation of latent virus .

In connection with these results it seems important the finding of increased virus neutralization antibodies as detected during the one year period. This fact was in good correlation with activation of latent virus.

The role of macrophages in the infection with some lymphotropic viruses is not well known. The infection of an organism with EBV usually causes inhibition of cell immunity and also the role of macrophages can be atypical. As far as EBV is concerned it was suggested that it replicates or is latent mostly in B-lymphocytes, but recently its relation to epithelial cells has been proved (Naher *et al.*, 1992). It is also known that CD4-lymphocytes contain the genome of EBV (Kikuta *et al.*, 1988). MHV-72 has a wide range of host cells for its replication (Svobodová *et al.*, 1982*a*).

Presented results proved that MHV-72 is a lymphotropic virus and a suitable model subject of study of pathogenesis of gammaherpesviruses, namely of EBV and *herpesvirus Saimiri*.It would be important to study intensively the molecular mechanism of latency of MHV and to investigate its association with lymphoma and hyperplasia of lymph nodes.

Our initial finding that female mice developed hyperplasia of cervical lymph nodes (about tenfold enlargement as compared to healthy mice) one and a half year after infection with MHV-72 (unpublished data), support our assumption that this virus might be a suitable model for the study of the oncogenic effect human lymphotropic gammaherpesviruses.

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