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PATHOGENESIS OF ACUTE AND PERSISTENT MURINE HERPESVIRUS INFECTION IN MICE

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Summary. — Outbred laboratory mice were inoculated at the age of 5, 10 and 21 days by oral and/or intranasal routes with 2 different (a lethal and a nonlethal) doses of the murine herpesvirus isolate 68 (MHV-68). Severe exudative pneumonia with haematogenous 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. Virus antigen was found by immunofluorescence (IF) in the alveolar lining of lungs, in heart muscle fibres, in spleen and thymic lymphocytes, in the tubular epithelium cells of kidneys, in the neurons of Gasserian ganglia and in the intima of large pulmonary vessels. Electron microscopy confirmed the transfer of virus particles through the capillary endothelium of the damaged alveolar septa. The surviving progeny and the mothers of animals, which had not succumbed to the lethal virus dose, were kept for 141—169 days when lungs and Gasserian ganglia were examined for virus presence. MHV-68 was recovered both by direct examination of the tissue homogenates as well as by the explantation technique. The results are suggestive for a dynamic persistence of MHV-68 rather than for static latency.

Key words: murine herpesvirus; pathogenesis; immunofluorescence; electron microscopy; histology; persistent infection

Introduction

Murine herpesviruses isolated from free-living small rodents (Blaškovič *et al.*, 1980) were studied by electron microscopy in rabbit embryo fibroblasts (Čiampor *et al.*, 1981). The growth characteristics in different cell cultures and the type of cytopathic effect as compared with mouse cytomegalovirus strain Smith allowed to classify these isolates as members of the subfamily *Alphaherpesvirinae* (Svobodová *et al.*, 1982*a*). This assumption was strengthened by serological assays comparing the isolates with the

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mouse cytomegalovirus and with some other alphaherpesviruses of man and animals (Svobodová *et al.*, 1982*b*). Because pathogenetic studies in experimental animals may be useful for comparison of viruses, outbred newborn mice were infected with lethal and sublethal doses of MHV-68 (Blaškovič, *et al.*, 1984).

The results showed that suckling mice developed severe pneumonia with dissemination of the virus to various organs including the CNS when inoculated with $\geqslant 10^3$ $TCID_{50}$ of the virus by oral route. The mothers of diseased offspring became inapparently infected as proved by reisolation of the virus from the explants of their trigeminal ganglia. Recent studies were designed to follow the experimental pathogenesis of MHV-68 infection in different age groups of young mice and to examine the nature of virus persistence.

Materials and Methods

Virus. MHV-68 was propagated in rabbit embryonic fibroblasts (REF) (kindly supplied by Řezáčová D., Institute of Sera and Vaccines, Prague). The titre of the stock virus was approximately 10^7 $TCID_{50}$/ml.

Animals. Families of outbred white laboratory mice (from the breed Velaz) born after shipment to the Institute were caged separately and infected by oral or intranasal routes as indicated in Table 1. The animals received either 10^4 or 10^3 $TCID_{50}$ of the virus in 0.02 ml or in 0.03 ml inocula. At given intervals, three mice were sacrificed and their organs were removed for virus titration, histology, immunofluorescence (IF) and electron microscopy (EM); the latter was performed only in 5-day-old mice inoculated with the higher virus dose. During the first 10—12 days post infection (p.i.) the mice were checked daily, later on once weekly for up to 5 months.

Virus titration. Blood was collected from the orbital sinus of the animals and mixed with a drop of heparin diluted 1 : 100 (final heparin concentration 2—4 μg/ml). Lungs, liver, kidneys, heart muscle and both Gasserian ganglia were removed to prepare 10% suspensions (v/w) in Eagle's basal medium (BEM) supplemented with 5—10% (w/w) of heat inactivated calf or bovine serum and antibiotics (100 units per ml penicillin and 100 μg/ml streptomycin). Tenfold dilutions were inoculated into REF cells grown in Leighton tubes in the same medium. The CPE was read 5—6 days after inoculation of the suspensions.

Immunofluorescence. The complex of lungs, heart and thymus, the liver and spleen, the complex of kidneys, suprarenal gland and lumbar cord, both Gasserian ganglia and salivary glands were removed at each interval and immediately frozen in liquid propanbutan. At least 20 and upmost 50 semiserial sections were cut from each block; the ganglia were cut in serial sections. The sections were fixed in acetone and stained with rabbit immune serum to MHV-68 diluted 1 : 10 (absorbed to suspensions of control mouse lung and liver) and with FITC-conjugated Sw-A-R/IgG (purchased from SEVAC, Prague) fractionated on DEAE-cellulose (the fraction eluted with 0.15 mol/l NaCl was used in a protein concentration of 2 mg/ml).

Electron microscopy. Lung samples were fixed in cold 2.5% glutaraldehyde in 0.2 mol/l sodium cacodylate buffer, pH 7.2, postfixed with 1% OSO_4 in the same buffer, dehydrated in an acetone series and embedded into Araldite. The blocks were cut in Pyramitome LKB. The thick sections were stained with toluidine blue to select the areas of exudation to the lung alveoli. After trimming in the Pyramitone, the elevated areas were cut in the Ultrotome III LKB. Ultrathin sections were stained with 2% uranylacetate and lead citrate and examined in a Philips EM 300 microscope at 80 kV.

Explantation technique. Lungs and Gasserian ganglia removed on days 141 and 169 p.i., respectively, were divided into 2 parts. One part was used to prepare the tissue homogenates or it was quickly frozen for IF studies. The other part was minced into small fragments, which were cultured for 8 days in BEM supplemented with 10% foetal calf serum and antibiotics. The medium fluid was exchanged on day 4. On day 8 the medium was removed, the fragments were washed in phosphate buffered saline pH 7.2, and quickly frozen. Cryostat sections were prepared

Table 1. Experimental design

Age (days)	Infectious dose	Inoculation route	Day p.i.	Examination	Lethality
5	10^4 $TCID_{50}$	oral	2, 4, 6, 8, 11	Hi, IF, EM	96%
	10^3 $TCID_{50}$	oral	4, 7, 11, 14, 27, 169	Hi, IF, VT, E*	18%
10	10^4 $TCID_{50}$	oral	4, 7, 11, 14, 27, 141	Hi, IF, VT, E*	20%
21	10^4 $TCID_{50}$	nasal	4, 15, 28, 141	Hi, IF, VT, E*	none

*E = explantation on days 169 or 141 only
Hi = histology, IF = immunofluorescence, EM = electron microscopy
VT = virus titration

by cutting the blocks of collected fragments originating from the same sample. The medium from both intervals was inoculated into REF cells.

Histology. The whole bodies of the 5-day-old animals or the organs of older mice removed from the pleural and abdominal cavities, their brain, spinal cord and Gasserian genglia were fixed in neutral formalin and embedded into paraffin. At least 10 and upmost 30 sections were prepared from each block and stained with haematoxylin and erythrosin (HE) and trichrome stain.

Results

Lethal infection of 5- and 10-day-old mice

The 5-day-old mice and a part of 10-day-old ones inoculated with the dose of 10^4 $TCID_{50}$ developed lethal disease between days 4—14 p.i. Histological examination of the lung tissue showed severe exudative pneumonia (since day 4 p.i.) in many sections (Fig. 1). The alveoli were filled with oedematic fluid, fibrin, macrophages and necrotic alveolar cells. Occasionally focal necrosis of the interalveolar septa was found (Fig. 2). Abundant lymphocytic infiltrates were seen in peribronchial connective tissue and in the septal wall. Focal necrosis of muscle fibers was present in the wall of heart ventricles associated with moderate mononuclear infiltrates at the edge of the lesions (Fig. 3). Minimal lymphocytic infiltration was present in portobiliary spaces of the liver. In meninges of the brain stem minimal round cell infiltration was seen. In the anterior horn of spinal cord small nodular infiltrates were present around a few neurons showing eosinophilic necrosis or tigrolysis.

The lung sections stained by IF revealed MHV-68 antigen in the alveolar wall, especially in the alveolar epithelium cells (Fig. 4). The positive fluorescence seemed to be present also in the wall of the capillary loops protruding into alveolar cavity from the interalveolar septa, but a clear-cut identification of virus antigen in the capillary endothelium was not possible. Occasionally positive staining was seen in the intima of larger pulmonary vessels including the endothelium (Fig. 5). Single hepatocytes showed positive

Table 2. Distribution of MHV-68 in 5-day-old mice infected with 10^3 PFU by oral route

Days p.i.	Blood VT	Lungs		Gasserian ganglia		Liver		Heart muscle		Kidneys	
		VT	IF	VT	IF	VT	IF	VT	IF	VT	IF
4	<1	4.5	++	0	0	2	0	0	0	<1	0
8	n.d.	4.5	++	<1	0	2.5	+	2.5	0	<1	+
11	n.d.	<1	+	<1	0	0	0	0	0	2.5	+
15	n.d.	4	+	3.5	+	3	+	4	+	4	+
30	n.d.	2.5	0	<1	0	2.5	+	3	+	2	+
169	n.d.	2.5[a]	+	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

VT = virus titre ($\log_{10}$ $TCID_{50}$/g tissue or 0.1 ml blood); IF = immunofluorescence (scoring: + = <5 sections positive out 50; ++ = ⩾ 6 sections positive)
[a] 4 out 5 animals positive (compare Table 5); n.d. — not done

fluorescence in the liver. Scattered single lymphocytes with ring-shaped cytoplasmic fluorescence were found in the spleen, thymus and bronchial lymph nodes. In the wall of heart ventricles a few muscle fibres contained the MHV-68 antigen. Single tubular epithelium cells and a few stromal cells were positive in the inner cortex of kidneys. These scattered findings of cells replicating the MHV-68 in different organs outside lungs were similar in both 5- and 10-day-old animals inoculated with the dose of 10^4 $TCID_{50}$. The lethal course depended on the extent of exudation into the lung alveoli (compare Figs 1 and 10).

Electron microscopic examination of the lungs of 5-day-old mice infected with the higher dose showed maximum lesions by day 9 p.i. Virus particles at different stage of assembly (naked capsids in the nucleus, enveloped particles in the cytoplasm) were seen predominantly in the alveolar epithelium cells (Figs 6, 7 and 8). In addition, occasional interstitium cells were found to replicate the virus. Virus particles were present within the cytoplasm of septal capillary endothelium. Extracellular enveloped particles were seen in the gaps between cells of the alveolar wall and within the alveolar cavity, which contained macrophages and polymorphonuclear leukocytes. The intercellular gaps between alveolar and capillary cells were widened. The endothelium cells and the alveolar epithelium cells showed heavy swelling, vacuolization and necrosis.

Nonlethal infection

About 80% of 5-day-old mice infected with the dose of 10^3 $TCID_{50}$ of MHV-68 and a similar proportion of 10-day-old mice infected with the dose of 10^4 $TCID_{50}$ survived for many weeks and was observed for 169 and 141 days, respectively (Table 1).

The virus distribution showed a good correlation as detected by titration and IF (Tables 2 and 3). The threshold for virus antigen visualization in semiserial sections seemed to be 10^2 $TCID_{50}$/g tissue. In semiserial sections

Table 3. Distribution of MHV-68 in 10-day-old mice

Days p.i.	Blood VT	Lungs		Gasserian ganglia		Liver		Heart muscle		Kidneys	
		VT	IF	VT	IF	VT	IF	VT	IF	VT	IF
4	<1	3	++	0	0	<1	0	0	0	0	0
7	n.d.	3	++	1.5	+	<1	+*	1.5	0	2.5	+
11	n.d.	3	+	2	+	0	0*	1.5	0	2.5	+
14	n.d.	2.5	n.d.	0	0	0	0	1.5	0	2.5	+
27	n.d.	3.5	+	0	0	<1	0	<1	0	<1	0
141	n.d.	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* positive lymphocytes in the spleen
For further explanations see Table 2

of the lung tissue stained by IF focal positivity of alveolar septi was occasionally found (Fig. 9). The MHV-68 antigen was also seen in stromal fibrocytes surrounding larger bronchi or vessels. The incidence of such cells was regarded for relatively frequent when one focus was present in at least 10—20% sections. At histological examination the foci showed exudation of fibrin into alveoli, thickening and lymphocytic infiltration of the septa (Fig. 10). At intervals since 11—15 days p.i., organization of fibrin and proliferation of the stromal connective tissue were found. The foci were surrounded with lymphocytary infiltrates.

Outside of the lungs single scattered parenchymal cells showing positive fluorescence were seen in the liver (Fig. 11), heart muscle and kidneys. In addition to tubular epithelium cells, a few positive stromal cells were found in the inner cortex of kidneys. Groups of a few lymphocytes with positive cytoplasmic fluorescence were detected in the red pulp of spleen (Fig. 12). Single positive neurons were observed in the trigeminal ganglion at different intervals from day 7 to 169 p.i.

In 21-day-old mice infected with 10^4 $TCID_{50}$ of MHV-68 lung tissue showed moderate virus replication especially at later intervals p.i. (Table 4).

Table 4. Distribution of MHV-68 in 21-day-old mice

Days p.i.	Lungs		Gasserian ganglia		Liver		Heart muscle		Kidneys	
	VT	IF	VT	IF	VT	IF	VT	IF	VT	IF
7	4.5	+	0	0	2	0	3	+	2	0
15	1.5	+	<1	0	0	0	<1	0	<1	0
28	1.5	+	<1	+[a]	0	0	0	0	<1	0
141	<1	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] 7 out of 2 000 ganglion cells positive
For further explanations see Table 2.

Table 5. Persistence of MHV-68 in mice

Days p.i.	Age at infection (days)	Virus dose ($TCID_{50}$ ml	Inculation route	Organs: Lungs			Gasserian ganglia		
				0	4*	8*	0	4*	8*
169	5	10^3	oral	4/5[a]	4/5	4/5	1/1	2/5	3/5
141	10	10^4	oral	6/6	6/6	6/6	n.d.	6/6	6/6
141	21	10^4	i.n.	5/5	5/5	5/5	n.d.	4/5	4/5
?	mothers	?	oral	2/3	3/3	2/3	1/1	2/3	2/3

[a] nominator: number of positive mice: denumerator: total number of mice tested
* Days in culture (explanted fragments): 0 — not cultured
? = infection of mothers acquired by contact
For further explanation see Table 2.

Nevertheless, small foci of positive fluorescence in the alveolar lining were present throughout. A few neurons showing MHV-68 antigen in their cytoplasm were found in serial sections of the Gasserian ganglion by day 28 p.i. (Fig. 13). Positive fluorescence in a few heart muscle fibres was seen by day 7.

Persistent infection of mice inoculated with the nonlethal virus dose

Surviving mice in each age group and 3 mothers of the succumbed progeny were examined on days 141 and 169 p.i. using the procedure indicated in Materials and Methods.

The number of mice whose lungs or Gasserian ganglia were positive for MHV-68 out of the total number of animals is shown in Table 5. It can be seen that all lung fragments and the great majority of the ganglion fragments yielded virus from the beginning of explantation procedure and that the non-cultured organ fragments were infectious as well. Morphological examination of the lung explants revealed the presence of the MHV-68 antigen in the desquamated alveolar cells lying within alveoli and in the stromal cells of the alveolar septa (Fig. 15). Occasionally small bronchi were filled out with mononuclear cells showing bright specific fluorescence. In the sections of the cultured trigeminal ganglia many neurons contained MHV-68 antigen in their nuclei and cytoplasm (compare Figs 13 and 14).

Discussion

According to its biological properties MHV-68 has been classified as the member of subfamily *Alphaherpesvirinae* (Svobodová *et al.*, 1982*a*). The animal host of this strain as well as of strains 60, 6 and 72 is *Clethrionomys glareolus*. Two another strains (Nos 76 and 78) have been isolated from *Apodemus flavicollis* from the same locality.

When inoculated into laboratory mice by oral or nasal routes, MHV-68 multiplied predominantly in lung alveolar cells. Several herpesviruses may grow in the lung alveolar cells as described with pseudorabies virus in piglets (Baskerville, 1973), rhinopneumonitis virus and cat rhinotracheitis virus (Love, 1971). From human herpesviruses varicella-zoster virus causes pneumonia in man, herpes simplex virus may cause penumonia in suckling mice (Rajčáni *et al.*, 1969). Experimental pneumonia was described in mice inoculated with human cytomegalovirus (Brody *et al.*, 1978). In the latter experiments, the necrotic endothelium of septal capillaries became leaky for peroxidase administered by intravenous route. Viraemia was demonstrated in newborn (Blaškovič *et al.*, 1984), in 5- and 10-day-old MHV-68-infected mice indicating the way of virus spread to spleen, liver, heart muscle and kidneys.

The neural spread and involvement of endoneural cells was repeatedly confirmed in newborn and young mice infected with herpes simplex virus (HSV) by various inoculation routes (Yamamoto *et al.*, 1965; 1968; Rajčáni *et al.*, 1969, 1970; Yamamoto *et al.*, 1973; Lascano *et al.*, 1980). The replication of HSV occurs in the Schwann cells of highly susceptible hosts in addition to the axonal spread, which may occur in the absence of virus growth in endoneural cells (Kristensson *et al.*, 1971; Cook and Stevens, 1973). Experiments are in progress to elucidate whether MHV-68 would spread to the sensory ganglia via axons. Nevertheless, MHV-68 antigen was not seen in Schwann cells in the sections investigated. As herpesviruses may reach the pseudounipolar neurons in the sensory ganglia also via bloodstream (Cook and Stevens, 1976), haematogenous spread of MHV-68 to the Gasserian ganglion seems highly probable.*

The persistence of HSV in experimentally infected mice and rabbits has been extensively documented (Baringer, 1975; Stevens, 1975; Klein, 1976). There was shown that herpes simplex virus resides in pseudounipolar neurons in a nonproductive form and it becomes activated upon recurrence or in the course of the explantation procedure (Kurata *et al.*, 1978; reviewed by Klein, 1982; Rajčáni and Szántó, 1983). When activated in the ganglion, herpes simplex virus spreads by a reversed axonal transport back to the inoculation site or to its close vicinity. The persistence of HSV at the inoculation site is not common, but it has been found in guinea pigs (Scriba and Tatzber, 1981). Our results had shown that MHV-68 persisted in a productive form in the portal of entry and in the sensory ganglia through the entire observation period of 5 months. The lung samples were infectious at their removal in each but one infected animal examined and the trigeminal ganglion samples yielded virus in 13 out 16 cases (Table 5).

Summing up, to characterize the pathogenesis of the MHV-68 infection in mice, the following conclusions can be drawn:

— MHV-68 persists in the lung tissue and sensory (trigeminal) ganglion in

* When mice were inoculated into the scarified cornea, we failed to demonstrate neural spread of MHV to the trigeminal ganglion.

Table 6. Herpesvirus persistence and latency in laboratory mice

Virus	Inoculation site*	Dominating spread	Regional ganglion	Persistence
HSV (human)	negative**	neural (axonal)	negative* (E positive)	static (nonproductive)
MHV (mouse)	positive	haematogenous	positive* (E positive)	dynamic (productive)

* direct virus isolation from the tissue homogenate at remote intervals ($\geqslant$ 30 days)
** during recurrencies intermittently positive (reversed axonal transport)
E — explantation

a productive form so that trace amounts of virus or single fluorescing cells can be found in non-cultured tissue samples;
— in acute disease MHV-68 spreads by haematogenous route crossing the damaged lung capillaries.

Based on the biological behaviour of the five MHV isolates and on their serological properties, they were found distinct from the murine cytomegalovirus strain Smith (Smith, 1954). This and previous studies were made in order to assess the pathogenesis of MHV and to support our conclusion to classify the MHV isolates as members of subfamily *Alphaherpesvirinae* rather then *Betaherpesvirinae* (Matthews, 1982). In general, the MHV-68 fulfilled the criterions defining the former subfamily: wide host range, a relatively short replication cycle, rapid spread in culture and establishment of latency in neural and nonneural tissues. Nevertheless, the distinction seemed uneasy as to the peculiarities of MHV latency and because of its predominantly haematogenous spread (Table 6).

Our study could also serve as a contribution to the epidemiology of herpesviruses under natural conditions. Identical viruses circulating in the same locality present in evolutionary distinct and unrelated animal species (*Clethrionomys glareolus* and *Apodemus flavicollis*) witness that these distinct species could be infected by the identical way, i.e. by ingesting the food contaminated with urine or oral secretions of any of these and may be of other rodent species. The horizontal way of infection occurred also in mouse mothers which had eaten their diseased offspring (Blaškovič *et al.*, 1984). The evidence that mothers overcame the infection with MHV-68 was proved by productive virus persistence in the lungs and in Gasserian ganglion.

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Explanation of Micrographs (Plates X—XV):

Fig. 1. Diffuse exudative pneumonia showing confluent necrotic foci: 5-day-old mouse, day 4 p.i., inoculated with 10^4 $TCID_{50}$ HE, ×40.

Fig. 2. Fibrin exudation, necrosis of septa, mononuclear and polymorphonuclear infiltration. The same mouse as in Fig. 1, HE, ×200.

Fig. 3. Necrosis of heart muscle fibres, mononuclear infiltration at the edge of lesions: 5-day-old mouse, day 4 p.i. inoculated with 10^4 $TCID_{50}$, HE, ×100.

Fig. 4. Positive IF of MHV-68 antigen in the wall of alveolar septi; indirect method. 5-day-old mouse inoculated with 10^4 $TCID_{50}$ by day 11 p.i., ×120.

Fig. 5. Positive fluorescence of MHV-68 in the intima of the pulmonary artery branch; elastic fibres counterstained with thiasin red. 5-day-old mouse, day 4 p.i., inoculated with 10^4 $TCID_{50}$, ×120.

Fig. 6. Alveolar septum at the edge of necrotic focus. MHV capsids in the nucleus and enveloped particles in the cytoplasm of an alveolar epithelium cell. The nucleus of the cell shows typical chromatin replacement to the edge of nuclear membrane, the cytoplasm shows vacuolization and loss of organelles. Enveloped particles in the gaps between septal cells and in the cytoplasm of the swollen capillary endothelium (CAP = capillary). Day 9 p.i., 5-day-old mouse, ×11 250.

Fig. 7. Detail of previous Figure showing the enveloped herpesvirus particles. ×40 000.

Fig. 8. Another area of the necrotic alveolar septum from the same mouse as in Fig. 6. Necrotic alveolar cell contains several capsids in the nucleus (arrowheads). In the swollen capillary endothelium a single enveloped particle can be seen (arrow); several enveloped particles in the cytoplasm of a type II alveolar cell (in the right). ER = erythrocyte. ×13 400.

Fig. 9. Focus of positive fluorescence of MHV-68 in alveolar and stromal cells: 10-day-old mouse, day 7 p.i., ×120.

Fig. 10. A medium sized focus of fibrin exudation found in semiserial sections; slight lymphocytic infiltration in the septi. Day 11 p.i., 10-day-old mouse, HE, ×100.

Fig. 11. A single hepatocyte shows positive IF in the nucleus and cytoplasm. Day 7 p.i., 10-day-old mouse, ×200.

Fig. 12. Scattered lymphocytes in the spleen red pulp showing ring-shaped positive fluorescence, day 5 p.i., 5-day-old mouse inoculated with 10^3 $TCID_{50}$.

Fig. 13. Positive fluorescence in the cytoplasm of a few neurons at different section levels of the same Gasserian ganglion; day 28 p.i., 21-day-old mouse, ×200.

Fig. 14. Positive IF in nuclei and cytoplasm of many pseudounipolar neurons (compare with Fig. 13). The Gasserian ganglion of a mouse infected by age of 10 days was removed on day 141 p.i. and subsequently cultured for 8 days. The collected ganglion fragments were cut in cryostat and stained by indirect IF; ×120.

Fig. 15. Positive IF in the content of alveoli (desquamated alveolar cells). The 5-day-old mouse infected with 10^3 $TCID_{50}$ of MHV-68 survived for 169 days. The lungs were cultured for 8 days and examinated by IF method; ×120.