

## EXPERIMENTAL PATHOGENESIS OF MURINE HERPESVIRUS IN NEWBORN MICE

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*Summary.* — Newborn white mice were susceptible to peroral (p.o.) infection with murine alphaherpesvirus isolated from free-living *Clethrionomys glareolus*. Death occurred within 6—8 days in animals infected with the higher virus dose of 4.8 log<sub>10</sub> TCID<sub>50</sub>. Clinical symptoms also occurred in some animals infected with lower doses, while others developed inapparent infection as judged by presence of humoral antibodies at 60 days post-infection (p.i.). The virus was detected in the lungs, blood, liver, spleen, kidneys, heart muscle, brain and urinary bladder of sick animals. Necrotising pneumonia accompanied the replication of the virus in the epithelial cells of alveolar ducts and alveolar lining as confirmed by immunofluorescence and histological examination. Latent infection of Gasserian ganglia in the survivors was not necessarily related to the administered dose of infectious virus. Two of mother females, which had eaten their diseased offspring, became inapparently infected as proved by reisolation of the virus from trigeminal ganglion explants and by detection of specific antibodies at 60 days p.i.

*Key words:* murine alphaherpesvirus; experimental pathogenesis; newborn mice; latency

### Introduction

Murine herpesviruses isolated from free-living small rodents (Blaškovič *et al.*, 1980) were preliminarily classified according to their biological characteristics as members of the subfamily *Alphaherpesvirinae* (Svobodová *et al.*, 1982a, b). An attempt was made at elucidating the pathogenesis of infection in newborn white mice using a strain of these viruses.

### Materials and Methods

*Virus.* The isolate No. 68 originating from *Clethrionomys glareolus* (Blaškovič *et al.*, 1980) was used. The virus was propagated in different cell cultures (Svobodová *et al.*, 1982a) reaching a titer of 10<sup>7</sup> TCD<sub>50</sub>/ml in a continuous line of rabbit embryo fibroblasts (REF).

*Cell cultures.* REF originating from the Institute of Sera and Vaccines, Prague, were used throughout. The cells were grown in basal Eagle's medium (BEM) supplemented with 5% of heat-inactivated bovine serum, with L-glutamine (3 g/l), 100 units of penicillin and 100 µg of streptomycin per ml. Virus titration was performed as indicated previously (Svobodová *et al.*, 1982a). REF monolayers (48–72 hr after seeding into test tubes) were inoculated either with 0.1 ml of organ suspensions or with the culture medium taken from infected REF. After 90 min adsorption, the medium was poured off, the cells were rinsed with 0.5% EDTA solution and afterwards fresh BEM was added. The medium containing virus from the first passage was inoculated in monolayers grown on coverslips in Leighton tubes; 3–10 days later, the cells were fixed either with formalin (for staining with haematoxylin-erythrosin, HE) or with acetone (for immunofluorescence staining).

*Experimental animals and their infection.* Outbred white pregnant mice and their newborns from the breed Dobrá Voda were used. Blood samples were taken from pregnant females before delivery and from their surviving offsprings 60 days post-infection (p. i.). Each infected and uninfected control mouse colony was kept separately and checked daily. During the experiment, the females had often eaten some of their sick progeny before this could have been virologically examined. Therefore baby mice, as soon as they developed symptoms of the disease, were sacrificed and examined either virologically or by histological or immunofluorescence techniques.

*Following organs were examined:* blood, lungs, liver, heart, brain, kidneys, spleen and urinary bladder. Those baby mice which did not become apparently ill and survived for 21 days p.i., were separated from their mothers and checked for virus presence in the Gasser ganglion 60 days p. i. At the same time interval some of the mothers, which had eaten their offspring, were also examined for virus persistence in the Gasserian ganglion as well as serologically. Newborn mice (6–9 in each family) were infected by peroral (p. o.) route. The animals received 0.8, 1.5, 2.8, 3.8 and 4.8 log<sub>10</sub> TCID<sub>50</sub> of the virus, respectively, in 0.02 ml inoculum. One colony of newborn mice was infected intracerebrally (i. c.) with 4.8 log<sub>10</sub> TCID<sub>50</sub> in 0.02 ml (positive control). The negative controls obtained 0.02 ml of uninfectious nutrient medium either p. o. or i. c.

The clinical symptoms were recorded daily. Sick individuals and their organs were examined for virus presence. Blood was sampled into heparin diluted in buffered saline (5 µg of heparin per ml). The urine was obtained by puncture of urinary bladder. From different organs 2.5–10% suspensions in nutrient medium were prepared; the suspensions were centrifuged (2000 rev/min for 10 min) and kept at –70 °C before inoculation into cell cultures. From i. c. inoculated mice, which all succumbed to infection, only brain suspensions were used for virus propagation.

*Preparation of hyperimmune serum* against the No. 68 virus and mouse cytomegalovirus and the virus neutralization test were described by Svobodová *et al.* (1982b).

*Fluorescent antibody (FA) staining* was performed as indicated by Svobodová *et al.*, 1982b). Lung and liver samples were quickly frozen in liquid propane-butane, cut in cryostat and fixed in acetone.

*Histological examination* of the organs followed the generally accepted techniques using paraffin-embedded blocks.

*Explants from Gasserian ganglion* for evidence of latency were prepared according to Rajčáni *et al.* (1975) and Sabó and Rajčáni (1976).

*Serological examinations.* Blood samples from pregnant females before delivery and 60 days after it and those from the infected progeny which survived for 60 days p. i., were taken from the sinus orbitalis. Virus neutralization test (VNT) using 10<sup>2</sup> TCID<sub>50</sub>/0.1 ml was used with all blood samples. Blood pool was prepared from 2–3 offsprings infected with low doses of virus. Special interest was given to the females which had eaten their offsprings. Blood samples from these females were tested individually.

## Results

### *Clinical symptoms in 1-day-old baby mice after the infection*

Control mice infected i.c. became ill on the 4th day. On the 5th day, one animal was found dead and the others, more seriously affected, were eaten by their mother during this day. The one-day-old baby mice infected p.o. (inhalation of the virus can be suggested) became ill or remained unaffected.

**Table 1.** Comparison of cytological examination and immunofluorescence in REF inoculated with suspensions from organs of newborn mice infected p.o. with 4.8 log<sub>10</sub> TCID<sub>50</sub>

Day p.i.	Staining	Organ							
		Lung	Liver	Blood	Heart	Brain	Kidney	Spleen	Urine
1	HE	+	0	0	0	0	0	0	0
	IF	+	0	0	0	0	0	0	0
2	HE	+	0	0	0	0	0	0	0
	IF	+	0	+	0	0	0	0	0
3	HE	+	±	±	0	0	0	0	0
	IF	+	+	±	0	0	0	0	0
4	HE	+	±	±	±	+	0	±	0
	IF	±	±	±	±	±	0	±	0
5	HE	+	+	+	+	+	+	+	0
	IF	±	±	+	±	+	0	+	0
6	HE	+	+	+	+	+	±	+	0
	IF	×	×	±	×	×	×	×	0
7	HE	+	+	+	+	+	+	+	0
	IF	+	+	+	+	+	+	+	0
8	HE	+	+	+	+	+	+	+	±
	IF	×	×	+	×	×	×	×	±
9	HE	+	+	+	+	+	+	0	0
	IF	+	+	+	+	+	+	0	0

HE = haematoxylin-erythrosin; IF = indirect immunofluorescence; ± = low degree of CPE or immunofluorescence in single cells only; + = virus presence proved; 0 = virus not detected; × = negative immunofluorescence with immune serum to mouse cytomegalovirus.

depending on the dose of virus administered. Those infected with 3.8 and 4.8 log<sub>10</sub> TCID<sub>50</sub>, respectively, became ill on the days 5–6 p.i. and died since day 8 or were eaten by their mothers. Newborn mice infected with 2.8 log<sub>10</sub> TCID<sub>50</sub> became ill on day 6 and showed symptoms of reduced mobility. In order to prevent cannibalism, they were killed on the same day. Newborn mice infected with 1.5 or 0.8 log<sub>10</sub> TCID<sub>50</sub> survived, showing no symptoms of disease.

All newborn mice inoculated with the control medium remained healthy and reached unaffected the age of 60 days, when the experiment was terminated. In all sick newborns the symptoms were very similar: weakness, loss of mobility, empty stomach. Reduced weight and growth as compared with healthy developing animals, shivering and cyanosis accompanied the signs mentioned above.

#### *Evidence of virus presence in different organs of infected newborn mice*

Virus presence in the investigated material was indicated by typical cytopathic effect (CPE) appearing on days 3–7 p.i. in the first passage and days 3–5 p.i. in the second passage. Rounding of cells, presence of naked nuclei, intranuclear Cowdry A type inclusions and sometimes polykaryocytes were observed.

Indirect immunofluorescence revealed the antigen in cytoplasm surrounding the nuclear membrane and in perinuclear region. No fluorescence was

Table 2. Virus titration in organ suspensions of suckling mice infected p.o. with  $10^{4.8}$  TCID<sub>50</sub>

Days p.i.	Organ							
	Lung	Liver	Blood	Heart	Brain	Kidney	Spleen	Urine
1	2.5	0	0	0	0	0	0	0
2	3	t	0	0	0	0	0	0
3	4.5	7	t	0	0	0	0	0
4	4.7	1.8	t	t	t	0	t	0
5	6.7	3.7	3.7	3.5	3.7	4	3	0
6	5.5	4	4	4.7	3.3	4.2	4	0
7	7	6.5	4.5	5	4.7	4.5	4	0
8	7.3	5.3	4.7	5.7	3.5	6.5	3	t
9	5	4	2.7	6.8	2	4.8	3.5	0

Virus titres are calculated per 0.1 g of the respective organ.

0 = virus was not detected in the 2nd passage.

t = traces (virus detected in the 2nd passage only).

seen when labelled immune serum against mouse cytomegalovirus was used. A comparison of both methods strongly confirming the presence of the virus in infected cells is presented in Table 1.

The titration results in organs of p.o. infected animals (Table 2) showed that the virus multiplied in the lungs since day 1 p.i. and thereafter spread to the liver, brain, kidneys and other organs probably via the blood stream. Newborn mice infected with the lower dose of  $1.5 \log_{10}$  TCID<sub>50</sub> showed virus clearance since day 5 with negative isolation results on days 8 and 10 p.i. (Table 3).

### *Morphological examinations*

As expected, pronounced lesions appeared in the lungs of animals receiving the highest virus dose. Scattered small foci of inflammation were seen on day 5 p.i. characterized by desquamation of epithelial cells, by presence of macrophages and exudation of oedematic fluid into alveoli. On days 7 and 8 p.i. necrotising pneumonia developed. Exsudation of oedematic fluid and accumulation of macrophages within alveoli were accompanied by desquamation of the alveolar lining (Fig. 1). Some epithelial cells of alveolar ducts and bronchioli revealed typical inclusion bodies. Focal necrosis of alveolar septa was prominent and was associated with mononuclear infiltration (Fig. 2). Positive fluorescence of the virus antigen occurred in epithelium cells of the alveolar duct, in alveolar lining and in interstitial cells of interalveolar septa (Fig. 3).

In the liver, mononuclear necrosis of hepatocytes with occasional intranuclear inclusion bodies was found (Fig. 4). The sinuses between trabecules were widened and contained macrophages, lymphocytes and the rests of extramarrow haemopoiesis (Fig. 5). Scattered positive fluorescence was observed mainly in Kupffer cells since day 6 p.i. (Fig. 6).

Table 3. Comparison of cytology and immunofluorescence in REF at 2nd passage<sup>a</sup>

Virus dose*	Day p.i.	Staining	Organ							
			Lung	Liver	Blood	Heart	Brain	Kidney	Spleen	Urine
1.5	1	HE	0	0	nd	0	0	0	0	nd
		IF	+	0	nd	±	0	0	0	nd
	2	HE	+	0	nd	±	0	0	0	nd
		IF	+	0	±	±	0	0	0	0
	3	HE	+	0	0	0	0	0	0	0
		IF	+	0	±	±	±	0	±	0
	4	HE	+	+	0	+	0	0	0	0
		IF	+	+	0	+	±	0	±	0
	5	HE	+	+	±	+	±	0	0	0
		IF	+	+	±	+	±	0	±	0
	8	HE	0	0	0	0	0	0	0	0
		IF	0	0	0	0	0	0	0	0
	10	HE	0	0	0	0	0	0	0	0
		IF	0	0	0	0	0	0	0	0
2.8	6	HE	+	+	±	+	+	0	+	0
		IF	+	±	+	0	+	0	+	0
3.8	8	HE	+	+	+	+	+	+	+	0
		IF	+	±	±	+	+	+	+	0

<sup>a</sup> REF were inoculated with the suspension of organs of newborn mice infected p.o. with different lower doses of the virus. Nd = not done. For further explanations see legend to Table 1.

\*  $\log_{10}$  TCID<sub>50</sub>

The lung tissue which had shown brilliant staining with homologous antiserum revealed very faint immunofluorescence when stained with antiserum to herpes simplex virus type 1.

#### *Latent virus in the explants of Gasserian ganglia*

Three mice surviving p.o. administration of  $1.5 \log_{10}$  TCID<sub>50</sub> were exsanguinated on day 60 p.i. No virus was recovered from explants of their Gasserian ganglia. Out of 9 offsprings receiving  $0.8 \log_{10}$  TCID<sub>50</sub> by p.o. route, one animal revealed latency, as detected by virus release from the ganglion explants into the medium of day 17 in culture. The REF inoculated with this medium sample in the second passage showed CPE by day 10 as confirmed by indirect immunofluorescence. Two of 3 females which had eaten their progeny (newborns given  $4.8 \log_{10}$  TCID<sub>50</sub> of the virus) showed latency in their Gasserian ganglia. This was proved by inoculation of the medium from ganglion explants to REF cells. In the latter, CPE developed slowly. The release of the virus from the ganglion explants was demonstrated as early as on day 10, and confirmed by FA staining.

#### *Serological evidence of infection*

Three offsprings surviving p.o. infection with  $1.5 \log_{10}$  TCID<sub>50</sub> had no NA antibodies as detected on day 60 p.i. One of 3 animals infected with  $0.8 \log_{10}$

TCID<sub>50</sub> by p.o. route revealed NA antibody in a titre of 4. Of the females, which had eaten their diseased offspring infected with the highest virus dose, one animal with proved latency of the virus in Gasserian ganglion developed NA antibodies in a titre of 64 (against 100 TCID<sub>50</sub> of the virus). Another female, not harbouring latent virus in its trigeminal ganglion, had antibodies in a titre of 4. No antibodies were found by neutralization tests in the blood of three other females investigated or in their offsprings.

### *Discussion*

One out of five herpesviruses (No. 68) isolated from free-living rodents was provisionally classified as member of the *Alphaherpesvirinae* subgroup (Blaškovič *et al.*, 1980; Svobodová *et al.*, 1982*a,b*). The criteria for this classification were in accordance with the internationally approved taxonomy (Matthews, 1982), in which the biological properties (growth in a variety of cell cultures of different animal species, pathogenicity for laboratory animals, type of the CPE etc.) represent the main differences between the subfamily of *Alphaherpesvirinae* and *Betaherpesvirinae*.

The reason to undertake the present study was to collect more information as to whether our preliminary classification of murine herpesviruses was correct and justified. There were no data available thus far that free-living rodents would harbour herpesviruses other than murine cytomegaloviruses. It has to be mentioned, however, that murine cytomegalovirus growth specificity for host animal cells in vitro cannot be decisive for classification of this virus to the genus (subfamily) of cytomegaloviruses (Kim and Carp, 1971). The course of infection with No. 68 murine herpesvirus in newborn white mice, i.e. in an animal phylogenetically related to the *Clethrionomys glareolus*, has shown that the pathways of virus spread in the organism after natural infection (p.o., i.n.) to a certain degree resemble to the pathogenesis of alphaherpesviruses in this laboratory animal (Rajčáni *et al.*, 1970). Several ways of virus spread should be considered, however; the blood dissemination and/or virus inhalation as well as the neural route complementing both. The latent infection of ganglion Gasseri, either productive or with the silent virus genome available for DNA transcription, resembles the natural and experimental latencies of alphaherpesviruses in neural tissue. It could be assumed that the performance of such experimental scheme may elucidate the natural ways of the alphaherpesvirus latency in this animal species. Experiments are in progress to study the nature of extraneural persistence of the virus in lung tissue. The state of virus latency in the Gasserian ganglion was established with the lower doses of ingested and/or inhaled virus. The symptomless multiplication of the virus was witnessed by induction of antibodies absent at the onset of the experiment.

Horizontal transmission of murine alphaherpesvirus was established by the infection of mother females which had eaten their infected progeny most probably when the mothers had recognized the disease in their babies. We suggest that the phenomenon of cannibalism of diseased offsprings by

their mothers is perhaps in some animals genetically determined in order to secure the development of a healthy generation. It should be mentioned that control newborn mice inoculated with nutrient medium developed no signs of injury, they suckled their mothers which did not harm them throughout the experiment. The result of this experiment has also proved the final occurrence of a persistent and latent infection in adult mice with alphaherpesviruses suggesting a — probably intraaxonal — spread of the virus to the pseudonipolar neurons of ganglion Gasserii. The consequence of this state of virus persistence, i.e. whether the excretion of the virus occurs into external environment, should be further elucidated.

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#### *Explanation of Micrographs (Plates XXXVI—XXXVII):*

- Fig. 1.* Lung of a suckling mouse infected with  $4.8 \log_{10}$  TCID<sub>50</sub> of the virus No. 68 on day 7 p.i. The onset of pneumonia with focal necroses of interalveolar septa; alveoli contain oedematous fluid, desquamated epithelial cells and macrophages.  $\times 150$ , (HE).
- Fig. 2.* Detail from Fig. 1: evident necrosis of interalveolar septa, in some cells typical intranuclear inclusions.  $\times 400$ .
- Fig. 3.* Positive fluorescence of virus antigen in the cells of alveolar epithelial lining and of alveolar septa. Also some cells in the lumen of alveoli reveal positive fluorescence. Day 7 p.i.,  $4.8 \log_{10}$  TCID<sub>50</sub>.  $\times 160$ .
- Fig. 4.* Intranuclear type A inclusion in a hepatocyte.  $\times 900$ .
- Fig. 5.* Monocellular necrosis and widening of sinuses in the liver, the intranuclear inclusion in a single hepatocyte enlarged (Fig. 4). Day 8 p.i.,  $4.8 \log_{10}$  TCID<sub>50</sub>.  $\times 400$ .
- Fig. 6.* Fluorescence of the antigen in single hepatocytes and in several Kupffer cells lining the sinuses; day 8 p.i.,  $4.8 \log_{10}$  TCID<sub>50</sub>.  $\times 200$ .