Acta virol. **31** : 25—30, 1986

CORNEAL INOCULATION OF MURINE HERPESVIRUS IN MICE: THE ABSENCE OF NEURAL SPREAD

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Received April 6, 1986

Summary. — Mouse herpesvirus (MHV) — a recently isolated herpesvirus — when inoculated into the right scarified cornea spread to lungs and liver by haematogenous route. Later on, the virus was recovered from lungs, spleen and trigeminal ganglia at intervals up to 8 months post-infection (p.i.). Another mice were infected by intranasal route and examined up to 264 days p.i. In latter case, MHV was recovered from lungs, spleen, kidneys, trigeminal ganglia and brain stem. At late intervals, direct isolation from lungs and spleen was as frequent as recovery of MHV from cultured explants; kidney fragments in culture yielded virus at a slightly enhanced rate, while positive MHV isolations from trigeminal ganglia and brain stem increased considerably after explantation. It is concluded that the persistence of MHV in lungs and spleen was productive with continuous presence of small but detectable amounts of infectious virus.

Key words: murine herpesvirus; persistent infection; latency; pathogenesis

Introduction

It has been shown that MHV strains isolated from wild-living rodents (Blaškovič *et al.*, 1980) cause lethal pneumonia and generalized infection in newborn laboratory mice (Blaškovič *et al.*, 1984). Persistent infection can be induced when older animals are infected by intranasal (i.n.) route (Rajčáni *et al.*, 1985). Previous studies indicated that MHV spread mainly via bloodstream. In addition, we found that virus persistence in lungs was of dynamic type, i.e. the virus could be isolated at any late interval p.i. by direct inoculation of the lung suspension onto REF cells. This was confirmed by

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isolation of a new MHV strain from the lungs of seropositive free-living *Apodemus flavicollis* species trapped in Šumava (Mistríková and Blaškovič, 1985). The purpose of present study was to extend the data concerning dynamic (productive) nature of MHV persistence and to gain more infomation on the route of virus spread to Gasserian ganglia.

Materials and Methods

Virus. Mouse herpesvirus strain 68 (MHV-68) isolated from *Clethrionomys glareolus* (Blaškovič *et al.*, 1980) has been used. The titre of stock virus suspension was 2×10^7 PFU/ml. The virus was propagated in rabbit embryonal fibroblasts (REF). The cells were grown in BEM supplemented with 5 % inactivated calf serum.

Animals. SPF white laboratory mice weighing 8—12 g were inoculated into right scarified cornea with 5 μl (10^4 PFU) and by intranasal (i.n.) route with 50 μl (10^5 PFU) undiluted stock virus suspension. The animals were kept under observation up to 9 months. Only occasional nonspecific deaths were recorded.

Virus titration. The organ homogeneates or those of cultured organ fragments were inoculated into REF monolayers grown in Leighton tubes (2×10^5 cells/ml). Lungs, liver, spleen, kidneys, both trigeminal ganglia, brain stem and forebrain were removed under sterile conditions. Blood was drawn from the ocular sinus. The intervals ranged from the acute stage (1—14 days p.i.) to 264 days p.i. (Tables 1, 2).

Explantation procedure. The organs were minced and small pieces (about 1 mm^3) were put on a sterile fabric in Petri dishes. The medium fluid (CMRL-1415 supplemented with 10 % foetal calf serum and antibiotics) was exchanged on cultivation days 3 and 7. The fragments from the same organ were collected and either homogenized or quickly frozen in liquid propanbutan.

Indirect immunofluorescence. Blocks prepared from organs removed at autopsy or those from collected explants were cut in cryostat and stained using rabbit immune serum to MHV-68 (kindly supplied by Dr. M. Stančeková) and anti-rabbit conjugate (Sw-AR, Sevac, Prague). Non-immune rabbit serum and the conjugate or the conjugate only were used to stain parallel (control) sections. In questionable cases, further parallel sections were stained with the serum adsorbed to uninfected organ suspensions. All sections were conterstained with 0.1 % thiasin red. At least 30 sections were cut from each block. The slides were viewed and photographed in microscope Jenalumar (Zeiss, Jena, G.D.R.).

Results

Virus spread after corneal inoculation

In mice inoculated into the right cornea MHV was isolated from homogeneates of the right cornea between days 3 — 14 p.i. (Table 1). At late intervals (87 — 220 days p.i.), no virus was isolated from the cornea and eye homogeneates; in one case it was recovered from the cultured eye fragments only (Table 2). In the course of acute keratitis MHV was occasionally isolated from blood, liver and lungs. Between day 1 to day 14 p.i. at least four mice were sacrificed; at each interval 1 pair of Gasserian ganglia was cultured. Neither the virus isolation from, nor the antigen detection in trigeminal ganglion sample of these animals was successful during acute keratitis. The results of ganglion and brain stem explantation were negative up to day 8 p.i.

Morphological studies revealed MHV antigen in sections of cornea on days 3, 5, and 8 p.i. and in the lung tissue by day 8 p.i. Positive fluorescence was

Table 1. Virus titration and immunofluorescence results after corneal infection

Organ	Days p. i.								
	1	3		4	5		7	8	14
	H	H	IF	H	H	IF	H	IF	H
Right cornea		1*	+	2	3	+	3	+	3
Blood		1			1				
Liver				1	1				
Lungs							1	+	
Spleen									
Kidneys									
RG, LG**									
Brain stem									
Brain cortex									

* Virus titre in 10% suspension (1 ml; $\log_{10}$ values); all other results were negative
** Right and left Gasserian ganglia were assayed directly and cultured for 7 days; the homogeneates of cultured fragments were inoculated into REF cells with negative results
H = homogenized samples; IF = immunofluorescence

present in corneal epithelium cells, in the fibrocytes of corneal stroma and of ciliary body (Fig. 1). Focal positive fluorescence was found in the lung alveolar epithelium cells in a few out of several sections.

Persistent and covert MHV infection

After corneal infection MHV persisted in the lungs and spleen where results of direct infectivity assay did not differ from the recovery rates found by explantation (Table 2). The Gasserian ganglion fragments yielded virus

Table 2. MHV isolation from tissue homogeneates in comparison to organ culture explants at late intervals post-infection into scarified cornea

Organ	Days p. i.					
	87—101		210—220		Total	
	H	E	H	E	H	E
Right cornea	0/8	0/7	0/9	1/10	0/17	1/17
Liver	0/8	n.d.	n.d.	n.d.		
Lungs	1/8*	2/7	4/9	4/9	5/17	6/16
Spleen	3/8	2/7	5/9	5/9	8/17	7/16
Kidneys	0/8	n.d	0/9	n.d.		
RG, LG	0/8	6/7	0/9	1/10	0/17	7/17
Brainstem	0/8	n.d.	0/9	n.d.		

* positive out total
H = homogeneate; E = explantation (in culture); RG, LG = right and left Gasserian ganglion

Table 3. Kineties of covert MHV activation in ganglion explants

Mouse no.	Sample	Inoculation route	Days in culture			
			3	7		Total
				M	F	
7	RG	right	1/7	4/7	3/7	7/7
	LG	cornea	0/7	0/7	2/7	2/7
7	RG	i. n.	0/7	0/7	3/7	3/7
	LG		1/7	4/7	3/7	7/7

F = fragments collected; they were either homogenized and assayed in REF cells or quickly frozen and examined by IF
M = medium fluid inoculated into REF cells

altogether in 7 out of 17 cases. In most samples, as seen in Table 3, the culture fluid was negative by day 3 and became positive by day 7 in culture. The virus was extremely rarely isolated from the right corneal fragments.

Similar conclusions were drawn from experiments in which mice were inoculated by i.n. route. In lung samples there was no difference between direct assay and virus recovery by explantation. Focal positive fluorescence of MHV antigen was seen in the alveolar wall, predominantly in alveolar epithelium cells (Fig. 2). In the kidney cut in semiserial sections, MHV antigen was found in the capillary endothelium cells of renal glomeruli (Fig. 3) as well as in the tubular epithelium cells (Fig. 4). The virus was recovered by direct assay also from renal homogeneates. The virus yield from explanted kidney fragments was slightly enhanced. No such difference between the frequency of direct assay and explantation results was noted in the spleen.

Table 4. Recovery of MHV from organs and tissue explants of mice at late intervals[a] after intranasal infection

Organ	Direct assay			Explantation		
	IF	H	Total	M	F	Total
Lungs	3/3	2/9	12/12	9/9	9/9	9/9
Lidneys	3/3	2/9	5/12	4/9	4/9	7/9*
Spleen	n.d.	4/6	4/5	4/6	5/6	5/6
RG, LG	0/3	2/5	2/8	4/7	3/7	7/7**
Brain stem	0/3	0/9	0/12	2/9	2/9	4/9

* in certain cases fragments were positive only, in another the virus was isolated from the culture fluid only, or the virus was recovered from both, M and F
** see also Table 3
IF = immunofluorescence; H = homogeneate; M = culture medium; F = fragments
[a] 240—261 days p. i.

In contrast, by direct assay the virus was significantly less frequently detected in trigeminal ganglion samples than in the ganglion ex plants (Table 4). From the latter, MHV was recovered after several days in culture indicating an activation kinetics closely resembling to herpes simplex virus (HSV) latency.

Discussion

Mice inoculated into the right scarified cornea with MHV developed viraemia between days 3 — 5 p.i. Occasional spread of the virus to liver and lungs has occurred on days 4 — 8 p.i. The virus was not found in trigeminal ganglia by direct assay during acute ocular disease and the explantation results were negative when the ganglia were removed by up to 8 days p.i. Explantation at early post-infection intervals would detect latency established by axonal transmission of HSV (Kristensson, 1970; Kristensson *et al.*, 1978). According to our experience in rabbits, otherwise undetectable amounts of HSV were recovered from ganglion explants already 18 — 24 hr after inoculation into scarified cornea (Rajčáni and Čiampor, 1978). Previous studies with MHV in newborn mice (Blaškovič *et al.*, 1984) did not show any involvement of Schwann cells, a hallmark of endoneural spread of HSV occurring under premissive conditions (Rajčáni and Conen, 1972; Lascano and Berria, 1980). Thus, in MHV-infected mice the neural spread seems unlikely to occur. Final proof should be given by footpad inoculation of MHV into mice with transsected sciatic and femoral nerves (Klein and De Stefano, 1983). On the other hand, latency was established with HSV also following viraemia after intravenous inoculation into tail vein (Cook and Stevens, 1976) and viraemia may occur as a consequence of HSV inoculation to cornea (Knotts *et al.*, 1974). We concluded, therefore, that MHV probably colonized the ganglia via bloodstream.

The second difference concerns the nature of covert ganglion infection (Rajčáni and Blaškovič, 1986). As already shown, the persistence of MHV in the lungs was of dynamic type. Similar results were obtained with spleen samples. Enhancement of virus isolation rate in kidneys from 25 % in direct assay to 77 % after explantation (Table 4) could indicate the inability to detect low amounts of infectious virus in direct assay (Roizman, 1965; 1974). For ganglion samples there was an even more significant enhancement of virus recovery rate by explantation procedure: in animals inoculated by i.n. route an elevation from 25 % to 100 %, and in those inoculated into the cornea an increase from none to 41 % was noticed. The activation kinetics in both animal groups resembled to that observed during culturing of ganglion fragments from mice with HSV latency. Our approach has demonstrated dynamic persistence of MHV in lung tissue, but in the case of chronic ganglion involvement it was difficult to discriminate between static and dynamic latency. The possibility that also nonproductive latency was established in MHV-infected ganglion cells cannot be exluded. Further studies employing nucleic acid hybridization techniques may be useful to solve this question.

Aknowledgements. The authors are thankful Dr. M. Stančeková for preparation of immune serum to MHV-68, Mrs. E. Srnová and Mrs. E. Trenčianská for their excellent technical assistance. The valuable suggestions of Prof. D. Blaškovič during the preparation of this manuscript are gratefully acknowledged.

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Explanation of Figures (Plate IV):

Fig. 1. Positive fluorescence of MHV antigen in the epithelium and stroma of ciliary body on day 8 after virus administration to scarified cornea.

Fig. 2. Focal fluorescence of alveolar epithelium cells in the lungs 240 days after intranasal inoculation of MHV; magn. ×120.

Fig. 3. Focal positive immunofluorescence of capillary endothelium cells in the glomerulus of renal cortex. Day 240 after i.n. infection; magn. ×300.

Fig. 4. Focal positive immunofluorescence of MHV antigen in tubular epithelium of renal cortex; day 240 after i.n. infection.