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Viremic dissemination of mouse hepatitis virus-JHM following intranasal inoculation of mice

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Summary. Using a sensitive infant mouse bioassay to detect infectious virus, the pattern of mouse hepatitis virus (MHV) JHM dissemination in blood and other tissues was examined during the first 5 days following intranasal inoculation. MHV replicated in nasal turbinates of both susceptible BALB and resistant SJL mice from days 1 through 5, but BALB mice had higher titers on days 1 and 2. Viremia was detectable on days 1 through 5 in BALB mice, but only on days 3 and 5 in SJL mice. Transient virus replication occurred in the lungs of both mouse genotypes at 1 and 2 days, then ceased. This correlated with more consistently demonstrable virus in blood collected from the left atrium of the heart, compared to jugular vein, portal vein and right atrial blood. Virus was associated equally with the plasma and cellular fractions of blood on day 3, but was primarily in the buffy coat of the cellular fraction on day 5. Interferon-α/β was detected in serum and spleen, but not liver or brain of BALB mice or in any tissue of SJL mice. BALB serum and spleen interferon was first detected at 36 h, peaked between 48 and 72 h, and was undetectable by 108 h. The distribution of virus in nose, cervical, axillary and mesenteric lymph nodes, spleen, Peyer's patch, thymus, bone marrow and liver was examined at 1, 2, and 3 days. The resulting pattern suggested lymphatic spread of virus to cervical lymph node and mesenteric lymph node as pathways of dissemination in addition to viremia.

Introduction

Mouse hepatitis virus (MHV) is a highly contagious and prevalent coronavirus of laboratory mice, with numerous related strains that partially differ antigenically, genetically and biologically [1, 23]. Like coronaviruses of other species, MHV strains display primary tropism for upper respiratory or enteric mucosa [1, 4]. In susceptible mice inoculated with respiratory-type MHV strains by the

intranasal (i.n.) route, virus spreads by direct extension from the nose to the brain [2, 9] and to other target organs such as liver and lymphoid tissue by a presumed viremic course. Viremia is suspected, since lesions and antigen are distributed in a vascular pattern [1, 4, 7, 18]. MHV viremia has been very difficult to demonstrate, since cell culture systems are relatively insensitive for detection of infectious virus in tissues. Despite evidence that MHV disseminates in a vascular distribution and infects blood-associated tissues such as bone marrow, lymphoid organs and vascular endothelium, viremia following i.n. MHV inoculation of immunocompetent mice has only recently been demonstrated by using an infant mouse infectivity assay. Under these circumstances, infectious virus could be detected in blood as early as 24 h after i.n. inoculation [5]. The purpose of the present study was to examine the early kinetics of viremia as the mechanism of MHV dissemination in genetically susceptible and resistant mice inoculated with a moderately virulent, respiratory-type strain of MHV.

Materials and methods

Experimental plan

The sequential appearance of MHV in respiratory tissues and blood was initially examined in genetically susceptible BALB/cByJ (BALB) and resistant SJL/J (SJL) mice following i.n. inoculation with moderately virulent MHV strain JHM. Previous studies have shown that both of these mouse strains develop disseminated infections between days 3 and 5 after i.n. inoculation, but virus titers and disease are significantly greater in BALB mice compared to SJL mice [5]. Groups of 5 randomly selected mice of each genotype were killed on days 1, 2, 3, 4, and 5 after inoculation. Infectious virus in nasal turbinates and lung on days 1, 2, 3, and 5, and blood on days 1, 2, 3, 4, and 5 was titrated. Sera were tested for detectable MHV antibody. To determine the relative rate of infection and MHV titers in each blood compartment, sets of blood samples were obtained from jugular vein, portal vein, left cardiac atrium and right cardiac atrium in a group of 6 BALB mice at 48 h after i.n. inoculation. Viremic blood samples from additional BALB mice on days 3 and 5 were pooled and differentially separated into cellular (erythrocyte and buffy coat) and plasma fractions. MHV titers were determined in whole blood, cellular and plasma fractions. The association of interferon-α/β with viremia was explored initially by analyzing pooled samples of nasal turbinate, blood, liver, brain, or spleen obtained from 3 mice of each genotype on days 0 (controls), 1, 2, 3, and 4 after i.n. inoculation. Based on findings from this experiment, interferon was assayed in individual samples of serum and spleen from 5 BALB mice per interval at 12, 24, 36, 48, 72, 84, 96, 108, and 120 h after i.n. inoculation. Finally, as another means of examining the routes of MHV dissemination, the sequential appearance of MHV in nose, cervical lymph nodes, axillary lymph nodes, mesenteric lymph nodes, spleen, Peyer's patches, thymus, bone marrow, and liver was examined in groups of BALB mice at 1, 2, and 3 days after i.n. inoculation.

Mice

Certified virus-free, 3–6 week old BALB and SJL mice were purchased from the Jackson Laboratory, Bar Harbor, ME and pregnant outbred CR1:CD1BR (CD1) mice were purchased from Charles River Laboratories, Portage, MI, shipped in filtered boxes, then transferred upon arrival into autoclaved micro-isolator containment cages (Lab Products,

Maywood, NJ) containing wood shavings, food (Prolab Animal Diet, Agway, Syracuse, NY) and water. Cages and mice were manipulated within a class II biological containment cabinet to preclude inadvertant exposure to adventitious murine viruses. Randomly selected mice were killed with carbon dioxide gas at specific intervals, and tissues were collected aseptically and frozen at −70 °C until tested for virus. Nasal turbinates were collected with forceps after removal of the dorsal nasal bones with a razor blade. Blood was collected in non-heparinized glass syringes and placed in vials containing EDTA as an anticoagulant. Peyer's patches consisted of intestinal mucosa containing gut associated lymphoid tissue. Blood was fractionated by centrifugation. Plasma was drawn off from the cellular fraction in separate aliquots. In additional samples, buffy coat was removed from erythrocytes, then erythrocytes were washed and centrifuged twice in saline.

Virus

MHV-JHM was obtained and maintained as previously described [5]. Mice were inoculated i.n. with 20 µl of cell-free culture fluid containing approximately 10^3 $TCID_{50}$ of MHV-JHM. Virus was detected in tissues by intracerebral inoculation of neonatal CD1 mice with 10% (w/v) tissue homogenates or whole blood in 0.025 ml volume. Virus titers were determined by similar inoculation of infant mice with serial 10-fold dilutions of tissue homogenates, and expressed as $\log_{10}$ LD_{50}/g, as previously described [5].

Interferon-α/β

Interferon-α/β was assayed in tissues by a cytopathic effect reduction assay, using mouse L929 cell monolayers challenged with 100 median infectious doses of vesicular stomatitis virus (Indiana serotype), as previously described [8, 15]. International units (IU) were determined with reference to a NIAID, WHO international reference standard (G002-904-511). Test tissues were triturated as 10% (w/v) homogenates, acidified to pH 2.0 overnight and neutralized prior to assay.

Antibody

Antibody to MHV-JHM was determined by enzyme immunoassay, using formalin-fixed MHV-JHM-infected 17Cl-1 cells as antigen and horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad, Richmond, CA) as described [17].

Statistical analysis

Differences in proportions were determind by χ^2 analysis and virus titers were compared with Student's paired or unpaired t tests.

Results

Respiratory tissues and viremia

MHV was detected in nasal turbinates of both BALB and SJL mice during the first 5 days after i.n. inoculation, but significantly higher titers were found in BALB mice compared to SJL mice on days 1 ($p \leqslant 0.05$) and 2 ($p \leqslant 0.01$) (Fig. 1). Viremia was detectable in BALB mice on days 1, 2, 3, 4, and 5, but only on days 3 and 5 in SJL mice (Fig. 1). Remarkably, MHV titers in blood on days 3 and 5 were equivalent between genotypes. MHV was detected in 1 of 4 BALB and 2 of 5 SJL lungs on day 1 and all of 5 BALB and 3 of 4 SJL lungs on day

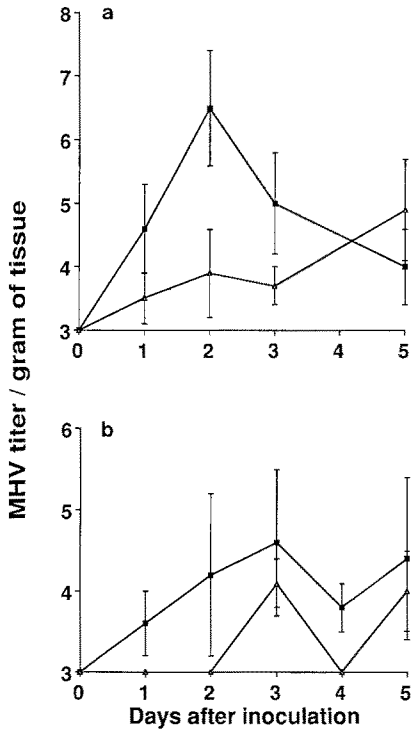

Fig. 1. MHV titers (log_{10} LD_{50}) in nasal turbinates (**a**) and blood (**b**) of BALB (− ■ −) and SJL (− △ −) mice at intervals after intranasal inoculation with MHV-JHM

2, but not at subsequent intervals (Table 1). MHV lung titers at 2 days were significantly higher ($p \leqslant 0.05$) in BALB compared to SJL mice.

Viremic blood compartments

Viremia was most consistently detected in blood samples taken from the left atrium compared to jugular vein, portal vein and right atrium at 2 days after i.n. inoculation (Table 2). Cellular and plasma fractions of BALB blood contained equivalent titers of MHV on day 3, but titers in plasma dropped significantly on day 5 relative to day 3 ($p \leqslant 0.05$) (Table 3). On day 5, the cellular fraction contained significantly more virus as the plasma ($p \leqslant 0.01$). In 4 of the 10 blood samples tested on day 5, virus could only be detected in the cellular

Table 1. MHV-JHM titers (log_{10} intracerebral LD_{50}/g) and rate of lung infection at intervals after intranasal inoculation of BALB and SJL mice

Genotype	Interval after inoculation (days)			
	1	2	3	5
BALB	3.2 (1/4)[a]	4.6 ± 0.6 (5/5)	(0/5)	(0/5)
SJL	3.4 ± 0.3 (2/5)	3.5 ± 0.2 (3/4)	(0/3)	ND

[a] Mean ± SD (number positive/number tested)
ND Not done

Table 2. MHV-JHM titers (log_{10} intracerebral LD_{50}) in blood and spleen of BALB mice at 48 h after intranasal inoculation

Mouse	Blood collection site				
	spleen	jugular vein	portal vein	left atrium	right atrium
1	4.9	3.6	–	3.9	–
2	4.6	–	3.2	3.6	3.2
3	4.9	3.2	3.2	3.6	–
4	4.9	–	3.2	3.9	3.6
5	3.6	–	–	3.9	3.2
6	4.6	3.2	3.9	3.6	–
Mean ± SD[a]	4.6 ± 0.5	3.3 ± 0.2	3.4 ± 0.4	3.8 ± 0.2	3.3 ± 0.2

– Undetectable (< 3.0 log_{10} LD_{50})
[a] Based on positive titers

Table 3. MHV titers in whole and fractionated blood on days 3 and 5 after intranasal inoculation of BALB mice with MHV-JHM

Interval (days)	n	Blood fraction		
		whole	cellular	plasma
3	5	4.1 ± 0.2[a]	4.5 ± 0.6	4.8 ± 0.6
5	10	4.0 ± 0.3	4.7 ± 0.7	2.3 ± 2.0

[a] Log_{10} LD_{50}/ml

fraction, and was consistently lower in plasma (3.5 ± 0.5 log_{10} LD_{50}) than in the cellular fraction (4.7 ± 0.7 log_{10} LD_{50}) in all 10 paired samples ($p \leqslant 0.001$). The cellular fraction was pooled from 4 mice and fractionated into buffy coat and erythrocyte fractions. Buffy coat contained 3.9 log_{10} LD_{50} MHV/ml, while erythrocytes had no detectable virus.

Interferon and antibody

Interferon-α/β was detected in pooled serum and spleen, but not nasal turbinate, liver or brain, of BALB mice and was not detected in any tissues of SJL mice. Levels in serum and spleen of BALB mice peaked at the 2 day interval. Based on these findings, interferon levels were assayed in serum and spleen of individual BALB mice (Table 4). Interferon titers were much higher in spleen compared to blood. In both tissues, interferon was first detectable at 36 h and peaked between 48 and 72 h. None was detectable by 108 h. MHV antibody was not detectable in sera of BALB or SJL mice through day 5.

Table 4. Titers and prevalence of detection of interferon in serum and spleen of BALB mice at intervals after intranasal inoculation with MHV-JHM

Interval (h)	Serum		Spleen	
12		(0/5)[a]		(0/5)
24		(0/3)		(0/5)
36	178	(1/5)	630 ± 0	(2/5)
48	178	(1/5)	3091 ± 1869	(5/5)
60	231 ± 288	(3/5)	4618 ± 2439	(5/5)
72	112 ± 63	(3/5)	4745 ± 3248	(5/5)
84	92 ± 89	(4/5)	316 ± 0	(4/5)
96	32 ± 0	(2/5)	1000 ± 0	(2/5)
108		(0/5)		(0/5)
120		(0/5)		(0/5)

[a] Mean titer ± SD, IU/ml of serum or gram of spleen (number positive/number tested)

Distribution of MHV in tissues of BALB mice

At 1 day after i.n. inoculation, infectious virus was detectable in nasal turbinate of all mice, in cervical lymph node in 4 of 12 mice, in spleen and Peyer's patch of a few others, but not other sites (Table 5). Thus, cervical lymph node sustained significantly higher rate of early infection compared to more distant axillary lymph node ($p \leqslant 0.05$), suggesting lymphatic spread of virus. By 2 days, MHV was present in nose, cervical lymph node, mesenteric lymph node and spleen of all mice; axillary lymph node, Peyer's patch and liver of over half of the mice; bone marrow of a few mice and not in thymus. Both cervical lymph node and mesenteric lymph node had higher rates of infection compared to axillary

Table 5. Rate of nasal, lymphoid tissue, bone marrow and liver infection at intervals after intranasal inoculation of BALB mice with MHV-JHM

Tissue	Interval after inoculation (days)		
	1	2	3
Nose	12/12[a]	24/24	12/12
Cervical lymph node	4/12	24/24	10/12
Axillary lymph node	0/12	16/24	10/12
Mesenteric lymph node	0/12	24/24	11/12
Spleen	3/12	24/24	11/12
Peyer's patch	1/12	21/24	10/12
Thymus	0/7	0/19	3/12
Bone marrow		4/14	8/12
Liver	0/12	13/24	8/12

[a] Number positive/number tested

lymph node ($p \leqslant 0.005$), suggesting lymphatic spread through both the head and gut. By 3 days, most tissues were infected in high prevalence, except thymus, which was positive in only 3 of 12 mice.

Discussion

Hepatitis and inflammation of other internal organs are important features of respiratory MHV infection in laboratory mice and are responsible for the name "hepatitis virus" that has been given to this group of murine agents. We now know that some MHV strains are strictly entertropic and seldom cause hepatitis regardless of the age or immune status of the host, while others, which replicate initially in nasal mucosa, can cause hepatitis if the virus is sufficiently virulent or the host is susceptible [1]. The polytropic, generalized nature of disease caused by these latter MHV strains in susceptible mice following i.n. inoculation is suggestive of viremic dissemination, but demonstration of MHV viremia has been inconsistent and usually following artificial routes of inoculation of immune-impaired mice with highly virulent strains of MHV. Viremia was detected with 17Cl-1 cell culture in C57BL/6 mice on day 2 and 3 after intracerebral (i.c.) inoculation with MHV-A59, but not following intraperitoneal (i.p.), i.n., or intragastric inoculation [12]. Viremia was demonstrable with DBT cell culture in 2 athymic BALB mice on day 14 after i.n. inoculation with wild-type MHV [7]. Swiss mice inoculated i.n. with MHV-S at 3 days of age had viremia detected in DBT cells on day 4, but not days 1, 2, or 3. Viremia could not be detected in older mice [18]. Much greater success was achieved in detecting and quantifying viremia as early as 12 h and through 5 days after i.p. inoculation of DDD and CDFl mice with virulent MHV-2, using DBT cell culture [19]. Highly virulent MHV-3 could be detected and quantified in the serum of 90 day old A mice on days 1, 4, and 5 after i.p. inoculation, using a mouse infectivity bioassay [13]. Likewise, virus titers in blood were monitored at several intervals up to 50 hours after intravenous inoculation of adult Swiss mice with MHV-3, using a mouse bioassay [14]. On the other hand, we and others [8, 9] have failed to detect viremia with cell culture assays following i.n. inoculation of adult mice with MHV-JHM. These inconsistencies are no doubt due to difficulties of growing nonadapted MHV in cell culture systems and their relative insensitivity for detecting infectious virus. We have found that the most sensitive means of detecting infectious MHV in tissues is the infant mouse bioassay and we have previously shown viremia following i.n. inoculation of young adult, immunocompetent mice with moderately virulent MHV [5] using the infant mouse bioassay that was employed in the current study.

The current results suggest that viremia is a very early event following i.n. inoculation, and can be detected within 24 h. Furthermore, lung appears to be an important early target for virus replication, and probably contributes to secondary viremia. MHV has been shown to replicate in pulmonary capillary endothelium and interstitium, with minimal infection of airway epithelium [3, 4, 7]. Virus titers in cardiac blood samples were highest in the left atrial samples,

compared to the right atrial, jugular or portal samples, supporting the pulmonary origin of some of the virus in the circulating blood. Fractionation studies of cardiac blood samples demonstrated infectious virus in both the plasma and cellular fractions on day 3, but virus had largely cleared from plasma by day 5, when it was associated with the buffy coat cells (leukocytes). MHV has a well known tropism for lymphoid tissue, as well as hematopoietic elements in bone marrow and spleen. The mechanism of plasma clearance by day 5 was not determined, but it was preceded by a drop in virus titer on day 4, which was apparent in both BALB and SJL mice. This biphasic pattern would suggest the influence of antibody or interferon, but antibody was not detected and serum interferon was present at low concentrations only in BALB mice. In a previous study, antibody was detectable on day 10, but not on day 5, in BALB mice and only 1 of 4 SJL mice tested on day 10 had detectable antibody [5].

The sequential distribution of MHV in parenchymal tissues suggests that virus dissemination also occurs through lymphatic drainage of the head, since cervical lymph nodes became infected earlier than other lymphoid tissues. This is not a significant event in MHV dissemination, since generalized involvement of virtually all target tissues has taken place by 3 days. The higher rate of mesenteric lymph node infection relative to axillary lymph node at 2 days suggests enteric entry as well. MHV-JHM, like most respiratory MHV strains, does not replicate to a very great extent in intestinal mucosa, but has a marked tropism for gut associated lymphoid tissue (GALT) [5]. The current study suggests early infection of GALT from the intestinal lumen, then mesenteric lymph nodes following i.n. inoculation. As previously demonstrated, this study confirms that lymphoid tissue in general is an important target for MHV. Involvement of the thymus in MHV-JHM infection has been reported in mice inoculated intracerebrally [10], but appears to be relatively rare following i.n. inoculation.

Interferon has been detected in serum, liver, spleen, and peritoneal exudate cells of mice infected with various MHV strains [8, 16, 19, 21, 22]. Correlation of interferon levels with susceptibility has been variable. Serum interferon concentrations were low in MHV-S infected, immature mice and elevated in adult mice [21]. Others [16, 19] have shown that resistant mouse genotypes (DDD, A/J) produce less serum and tissue interferon than susceptible genotypes (CDF1, C57BL) when infected with MHV-2 or MHV-3. No differences in serum interferon concentrations were detected between susceptible (C3H, BALB) and resistant (DDD, CF1) mice infected with MHV-3 or MHV-JHM [8, 25], although peak interferon titers occurred later in susceptible BALB compared to resistant CF1 mice [8]. In the current study, interferon was found in spleen and serum of MHV-JHM-infected BALB mice, but not nose, liver or brain and was not detectable in any tissue of SJL mice. Since all of these tissues were infected in both genotypes, as demonstrated previously [5], interferon had no correlation with presence, clearance or absence of infectious MHV. These data,

and those of others, indicate that serum or tissue interferon responses of mice to MHV vary greatly with mouse genotype. Furthermore, MHV strains vary in their sensitivity to interferon in vitro [8, 20].

SJL mice are well known to possess remarkable resistance to MHV disease. They have been shown to lack a functional cellular receptor for MHV-A59 in at least some target tissues [6, 24]. We have previously demonstrated that SJL mice develop very mild infections of liver, spleen and lymphoid tissue compared to BALB mice, when inoculated by the i.n, route [5]. The current study demonstrates that SJL mice support virus replication in their nasal turbinates and also develop a demonstrable viremia, albeit intermittent. Thus, their resistance to MHV-JHM-induced disease is not a reflection of their absolute resistance to infection or ability to support virus replication.

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