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Olfactory neural pathway in mouse hepatitis virus nasoencephalitis*

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Summary. The mechanism of brain infection with mouse hepatitis virus-JHM was studied in BALB/cByJ mice following intranasal inoculation, and found to be a consequence of direct viral spread along olfactory nerves into olfactory bulbs of the brain. Infection was followed sequentially from nose to brain, using microscopy, immunohistochemistry and virus quantification. Lesions, antigen and virus were observed in the olfactory bulb and anterior brain as early as 2 days and posterior brain by 4 days after inoculation. Viral antigen extended through nasal mucosa into submucosa, then coursed along the olfactory nerve perineurium and fibers, through the cribriform plate into the olfactory bulbs. On days 4 and 7, viral antigen was found in the antero-ventral brain, along ventral meninges, olfactory tracts and anterior ramifications of the lateral ventricles. Virus was cleared from nose by 10 days and anterior brain by 20 days, but persisted in posterior brain for 20 days after inoculation. Mice also developed disseminated infection, with viremia and hepatitis. Infection of brain did not correlate with presence of viremia. In contrast to intranasally inoculated mice, orally-inoculated mice did not develop encephalitis, despite evidence of disseminated infection.

Key words: Mouse hepatitis virus – Nose – Brain

The murine coronavirus, mouse hepatitis virus (MHV), has served prominently as a model of viral encephalitis and demyelinating disease in mice and rats [7, 18–20]. Although most investigations have utilized intracerebral inoculation to induce central nervous system disease, several strains of MHV readily infect brain following intranasal (i.n.) inoculation [1, 2, 6, 8, 10, 13, 14, 17]. Following initial replication in the primary target tissue (nasal epithelium), MHV appears in olfactory pathways within the brain [2, 6, 8, 14, 17]. Although evidence is circumstantially strong for direct extension of MHV from the nose into the brain, it has not been conclusively proven. The purpose of this study was to determine the route of virus entry into brain by examination of the sequential progression of infection with neurotropic MHV-JHM following i.n. inoculation of mice.

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Material and methods

Mice

Three-week-old BALB/cByJ mice were purchased from the Jackson Laboratory (Bar Harbor, Me) and Crl:CD1BR mice were purchased from Charles River Laboratories (Portage, Mich). Both sources were MHV free. Mice were shipped in filtered boxes and transferred on arrival into sterile Micro-isolator (Lab Products) cages containing pine shavings, food (Purina Laboratory Chow) and water. Mice were inoculated i.n. or per os (p.o.) with 10 μl of cell-free culture fluid containing 10^3 median tissue culture infectious doses of MHV-JHM.

Virus

MHV-JHM was obtained from the American Type Culture Collection (Bethesda, Md), passaged twice in NCTC-1469 cells, once in BALB/cByJ brain and once in 17 Cl 1 cells [16]. Infectious virus was quantified in tissues by infant mouse infectivity assay. Frozen tissues were thawed, weighed and diluted 10% (w/v) in Dulbecco's minimal essential medium containing 5% fetal bovine serum. They were homogenized, then clarified in a refrigerated centrifuge at 2,000 rpm for 20 min. Serial tenfold dilutions of supernates in 0.025 ml were inoculated intracerebrally into 2-day-old Crl:CD1BR mice in groups of four dilution. Mortality was established at 72 h and the $\log_{10}LD_{50}$/gram of tissue was determined [15].

Histology/immunohistochemistry

Tissues were immersion fixed in neutral buffered formalin, pH 7.2, and paraffin embedded. Skulls were demineralized in a solution containing 1.8 g tetrasodium ethylenediaminetetraacetic acid, 0.7 g sodium tartrate, 125 ml hydrochloric acid and 875 ml distilled water. Tissues were sectioned at 5 μm and stained with hematoxylin and eosin. Selected tissues were stained for MHV antigen using a streptavidin-biotin-horseradish peroxidase (Bethesda Research Laboratories) method and tissue treatment as previously described [5]. Primary antibody was hyperimmune mouse ascitic fluid prepared in multiparous Crl:CD1BR mice by 3-weekly intraperitoneal injections of

MHV-JHM-infected infant mouse brain emulsified in Freund's complete adjuvant.

Tissue samples

Groups of five BALB mice were killed at intervals (3 and 12 h; 1, 2, 3, 4, 5, 7, 10 and 20 days) after i.n. MHV-JHM inoculation. Mice were randomly selected for necropsy and killed with carbon dioxide gas followed by cardiac exsanguination. Following removal of the lower jaw and skin, the head was sagitally hemisected. One hemisection was placed in formalin. The nasal turbinates and brain were removed for virus recovery from the other hemisection. The brain was then cut coronally into anterior and posterior segments. Nose, olfactory bulb, brain, and liver were collected for histology and immunohistochemistry. Nasal turbinate, anterior brain, posterior brain and whole blood were collected and frozen at −70° C for virus determination. In addition, groups of five BALB mice were inoculated i.n. or p.o. and the subsequent patterns of infection in nose, brain and liver were compared microscopically on day 5 after inoculation.

Results

Histology/immunohistochemistry

The distribution of lesions suggested a sequential progression of infection from nose to posterior brain (Fig. 1). Necrosis of nasal respiratory and olfactory epithelium was observed as early as 12 h after MHV inoculation, with extension into submucosa by 24 h. By day 2, necrotizing lesions were visible in the anterior olfactory bulb. At subsequent intervals, necrotizing inflammation extended posteriorly along the meninges and olfactory tracts of the anteroventral brain, as well as into the rostral ramifications of the lateral ventricles and hippocampus. Involvement of the posterior brain was first evident on day 4. On days 10 and 20, necrotizing changes were absent, but mild gliosis and nonsuppurative meningitis were present in areas lytically affected at previous intervals. Livers from all mice on days 4 and 6 had multifocal necrotizing hepatitis.

The presence of MHV antigen correlated with microscopic lesions in target tissues in the early phase of infection (Fig. 1). In the anterior nose, MHV antigen was present in ciliated respiratory epithelial cells as early as 24 h after inoculation (Fig. 2A). In the posterior nose, MHV antigen was present in olfactory epithelial cells, as well as within submucosal tissues by 24 h (Fig. 2B). Antigen appeared to extend along olfactory nerve perineurium (Fig. 2C) and to a lesser extent, within nerve fibers (Fig. 2D), often in areas without involvement of overlying mucosa. Antigen was present around nerve fibers as they penetrated the ethmoid cribriform plate (Fig. 2E) and appeared within the olfactory bulb meninges and cells of the glomerular layer within 48 h (Fig. 2F). Antigen also appeared in anterior brain at this interval. On days 4 and 7, MHV antigen was evident in anteroventral

TISSUE	DAYS AFTER INOCULATION									
	0.5	1	2	3	4	5	6	7	10	20
	MHV HISTOPATHOLOGY									
Nasal mucosa	■	■	■	■	■	■	■	■	□	□
Nasal submucosa	□	■	■	■	■	■	■	■	□	□
Olfactory bulb	□	□	■	□	■	■	■	■	□	□
Anterior brain		□	□	□	■	■	■	■	■	■
Posterior brain		□	□	□	■	■	■	■	■	■
	MHV ANTIGEN									
Nasal mucosa		■	■		■			■	□	
Nasal submucosa		■	■		■			■	□	
Olfactory bulb		□	■		■			■	□	
Anterior brain		□	■		■			■		
Posterior brain		□	□		■			■		

Fig. 1. Distribution of mouse hepatitis virus (MHV) histopathology and antigen at intervals after intranasal (i.n.) MHV-JHM inoculation of mice. ■ positive, □ negative

meninges (Fig. 3A), ependyma of the rostral lateral ventricles (Fig. 3B) and brain (Fig. 3C). Brain appeared to be infected by spread from meninges and ventricles, as well as by extension along olfactory tracts. Antigen was seen nonselectively in meningeal, neuronal, glial and ependymal cells.

Virus

Virus detection confirmed morphological impressions of an anterior to posterior progression of MHV from nose to posterior brain, as well as anterior to posterior regression of infection (Fig. 4). At 3 h after i.n. inoculation, only one of five mice had detectable virus in nasal tissue, which probably represented residual inoculum. By 12 h, all mice had demonstrable virus in nasal tissue, which rose in titer through day 2, then declined. Virus was first detected in anterior brain on day 2 after inoculation and cleared by day 20. In contrast, virus was not detected in posterior brain until day 4 and was still present in brains of all infected mice at day 20. Viremia was confirmed in four of five infected mice at 24 h and all mice at 2, 3, 4 and 5 days after i.n. inoculation.

Intranasal versus oral inoculation

To confirm that brain infection resulted from direct extension of virus along olfactory nerves and not due to viremia, groups of five mice were inoculated either i.n. or p.o. with MHV-JHM. At 5 days after inoculation, the distribution of MHV lesions was compared between the two groups (Table 1). Both groups developed the same prevalence and severity of hepatitis,

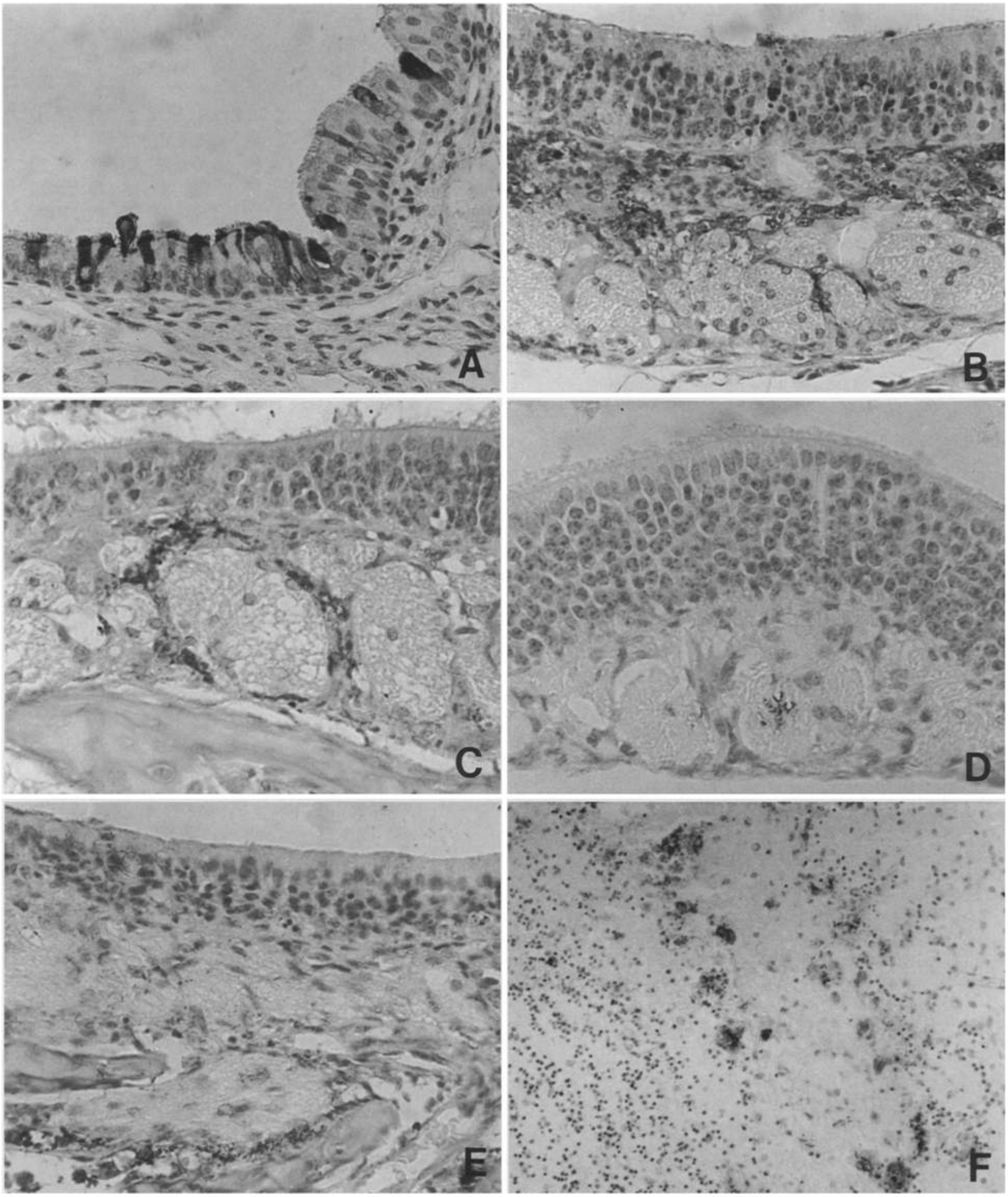

Fig. 2A–F. MHV immunoperoxidase staining of tissues after i.n. MHV-JHM inoculation of mice. **A** Antigen in ciliated respiratory epithelial cells of anterior nose; **B** Antigen in olfactory epithelium and submucosa of posterior nose; **C** Antigen in olfactory nerve perineurium within nasal submucosa; **D** Antigen in olfactory nerve fiber within submucosa of posterior nose; **E** Antigen in perineurium of an olfactory nerve as it penetrates the ethmoid cribriform plate; **F** antigen in cells of the olfactory bulb lamina glomerulosa; **A–E** ×375, **F** ×180

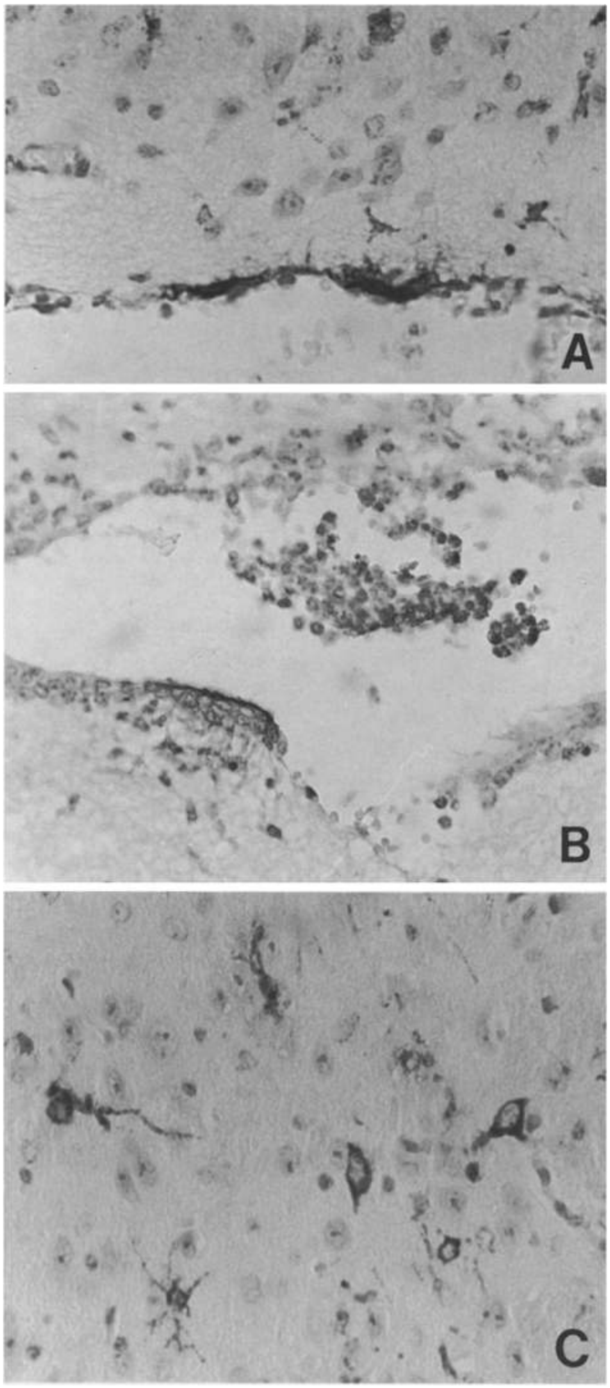

Fig. 3A–C. MHV immunoperoxidase staining of brain after i.n. MHV-JHM inoculation of mice. **A** Antigen in leptomeninges and glial cells of adjacent parenchyma in the anteroventral brain; **B** antigen in intact and detached ependymal cells of the anterior lateral ventricle; **C** antigen in cytoplasm of neuronal and glial cell bodies and processes in the posterior brain stem. **A–C** ×375

indicative of viremic dissemination. However, only i.n.-inoculated mice developed brain lesions. Interestingly, orally inoculated mice had MHV lesions in the anterior nose involving respiratory epithelium,

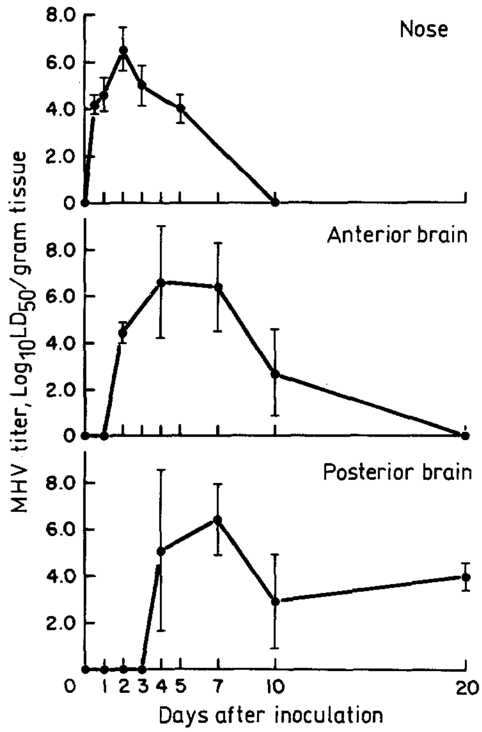

Fig. 4. MHV titers in nasal tissue, anterior brain and posterior brain at intervals after i.n. MHV-JHM inoculation of mice

Table 1. Comparison of mouse hepatitis virus (MHV) lesion prevalence in selected target organs of mice inoculated intranasally or orally with MHV-JHM

Organ	Route of inoculation		Chi-square *P* value
	Intranasal	Oral	
Anterior nose	5/5[a]	5/5	n.s.d.
Posterior nose	5/5	0/5	< 0.001
Olfactory bulb	5/5	0/5	< 0.001
Brain	5/5	0/5	< 0.001
Liver	5/5	5/5	n.s.d.

[a] 5 positive/5 examined
n.s.d.: No significant difference

but not in the olfactory epithelium of posterior nasal tissue.

Discussion

This study provides insight into one mechanism of central nervous system infection by viruses with respiratory tropism. Mouse hepatitis virus, similar to coronaviruses of other species, utilizes upper respiratory mucosa as its primary target [2, 4, 6, 8, 17]. A number of studies have demonstrated olfactory bulb or tract lesions following i.n. inoculation of different MHV strains [1–3, 6, 8, 10, 13, 17]. Results of the present study provide evidence that virus enters the brain directly by extension along olfactory nerves. Sequential studies of lesion, antigen and virus distribution in nose and brain indicated anterior to posterior progression of infection. Furthermore, mice inoculated orally with MHV did not develop brain infection, although they showed evidence of viremic dissemination to liver. This suggests that there is an effective blood-brain barrier to MHV in mature mice. In contrast, the pattern of brain infection is decidedly different in neonatal mice. In these animals, MHV lesions are diffusely distributed throughout the brain, suggesting blood-borne infection [3, 4]. The mechanism of this age-dependent blood-brain MHV barrier is worthy of further investigation. Orally inoculated mice developed MHV lesions in their anterior nasal tissues, presumably by entry of virus through the incisive foramina, patent openings between the mouth and anterior nose [9]. The fact that none of these mice developed brain infections despite anterior nasal infection suggests that olfactory neural extension of virus is inefficient and requires infection of the posterior nasal tissues, where olfactory epithelium and nerves prevail.

Other studies have indicated that among several prototype MHV strains tested, all strains infected anterior olfactory pathways of brain following i.n. inoculation of adult mice. However, only those virus strains with relative neurotropism were capable of progressing into the posterior brain [6]. Thus, progression of infection within brain is MHV strain specific. The sequential distribution of lesions within the brain up to 7 days after i.n. inoculation of neurotropic MHV-JHM has been well described [8], and resembled the posterior progression seen in our studies. The present study examined infection only for 20 days, but showed a sequential anterior-posterior regression of lesions and virus within the brain. Persistence of low levels of virus in the posterior brain stem corresponded with the location of persistent spongiform and demyelinating lesions in the brain stem following i.n. inoculation of several different strains of MHV [1, 2, 4, 6]. Although MHV-JHM is usually cleared from brain by 60 days after i.n. inoculation of susceptible mice [4], temperature-sensitive mutants of MHV-JHM have been shown to persist for up to one year after intracerebral inoculation [11, 12].

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