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ORGAN SPECIFIC ENDOTHELIAL CELL HETEROGENEITY INFLUENCES DIFFERENTIAL REPLICATION AND CYTOPATHOGENICITY OF MHV-3 AND MHV-4

Implications in Viral Tropism

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INTRODUCTION

The various strains of mouse hepatitis virus exhibit distinct organ tropisms. MHV-4(JHM) and MHV-3 are predominantly neurotropic and hepatotropic respectively. Studies on the mechanisms involved in organ specific infection of mouse hepatitis virus (MHV) have focused on several factors such as dose, route of administration, age and strain of experimental animals[1]. Another potential mechanism for regulation of tissue specific spread of virus is the ability of organ specific endothelial cells to selectively support the replication of different strains of MHV. Endothelial cells form an interface between circulating blood and organs and thus could serve as a barrier to infection by viruses. Endothelial cells are also heterogeneous in different organs and may exhibit selectivity in permitting viral entry, thus influencing their tissue tropism[2].

In order to study the role of endothelial cells in regulating viral tropisms, primary cultures of hepatic endothelial cells, hepatocytes and cerebral endothelial cells derived from MHV susceptible BALB/c mice were infected with MHV-3 or MHV-4. Supernatants from infected cultures were collected, for performing plaque assays, at various time points after infection. Cytopathic effects were also monitored at these time points.

The data presented indicates that the endothelial cells but not hepatocytes, demonstrate differential responses to MHV-4 and MHV-3 infection. Endothelial cell heterogeneity thus could play an important role in regulating organ tropism of MHV-3 and MHV-4. The possible mechanisms of differential replication of MHV strains in organ specific endothelial cells are discussed.

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MATERIALS AND METHODS

Brain Derived Endothelial Cell Cultures

Cerebral microvascular endothelial cells (CEC), were isolated from the brains of BALB/c mice (2 months old) using the method described by Rupnick et al[3]. Briefly, cerebral microvessels were isolated by digestion of cerebral white matter in 0.5% collagenase (Sigma, St.Louis, MO), and density centrifugation of homogenized material in 15% dextran. The vascular pellet was further purified by gradient centrifugation on Percoll(45%). Capillaries were plated onto 0.1% gelatin-coated plates, and endothelial cells grew out in about 10 days. Endothelial cell lines were established from these initial outgrowths and were cultured in Medium 199 (GIBCO, Grand Island, NY), supplemented with 20% fetal bovine serum, 2mM L-glutamine, 90μg/ml heparin (porcine, Sigma, St. Louis, MO), and 20μg/ml endothelial cell growth factor (ECGF, Collaborative Research, Cambridge, MA).

Hepatic Endothelial Cell Cultures

Hepatic endothelial cells from BALB/c mice (2 months old) were isolated using a method described by Huber et al[4]. Liver tissues were minced and digested in 0.5% collagenase and homogenized. The homogenized material was spun at 160 x g to pellet the cells. The cell pellet was centrifuged at 400 x g for 15 minutes on a gradient consisting of 29%,37% and 55% isotonic Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). Hepatic endothelial cells were found between the 37% and 55% Percoll interface. The endothelial cells were washed and plated in the medium utilized above for cerebral microvascular endothelial cells. Endothelial cell identity for all sources was established by the uptake of DiI-Ac-LDL (acetylated low density lipoprotein, Biomedical Technologies, Stoughton, MA), and specific binding of the lectin *Bandeiraea simplicifolia* BSI-B_4[5,6]. The endothelial cell lines used in these studies were between passages 8-12.

Hepatocyte Cultures

Primary hepatocyte cultures were established from BALB/c mice (2 months old) as described by Seglen[7]. Hepatocytes were isolated by collagenase perfusion (Type I, Sigma, St. Louis, MO, 130U/ml) through the portal vein and plated (5 X 10^5 cells/T25 flask) in complete Williams E medium (GIBCO Laboratories, Chagrin falls, OH) containing 10IU/ml penicillin, 10μg/ml gentamycin, 0.02U/ml insulin and 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA).

Virus Infection of Cultures

Endothelial cells and hepatocytes grown in T-25 flasks (5 x 10^5 cells/flask) were infected with MHV-3 or MHV-4 at an MOI of 0.1. MHV-4 (obtained from Dr. Robert Knobler) was grown to a stock containing 3 x 10^6 PFU/ml. MHV-3 (ATCC) was grown to a stock containing 6 x 10^6PFU/ml. At various times after infection (24, 48, 72 and 96 hours) culture supernatants were collected for measuring viral titers. At these time points cytopathic effects were also documented. Virus titers in the supernatants were measured by plaque assays on L-2 cells.

RESULTS

Comparison of MHV-3 and MHV-4 Effects on Hepatic Endothelial Cells

Fig. 1 compares the cytopathic effects on hepatic endothelial cells at 24, 48, 72 and 96 hours following treatment with MHV-3 or MHV-4. The hepatotropic MHV-3 causes syncitium formation and extensive cellular destruction on hepatic endothelial cells as early as 24 hours following infection (Panel A). In contrast the neurotropic MHV-4 infection does not result in syncytium formation or cause other cytopathic effects on hepatic endothelial cells at 24 hours (Panel E). Interestingly, the earliest evidence of any cytopathology (cellular granularity) was seen only at 96 hours after infection with MHV-4 (Panel D).

Comparison of MHV-3 and MHV-4 Effects on Hepatocytes

Fig. 2 compares the cytopathic effects on hepatocytes at 24, 48, 72 and 96 hours following infection with the hepatotropic MHV-3 or neurotropic MHV-4. Unlike the findings obtained with hepatic endothelial cells both virus strains caused equivalent cytopathic effects on hepatocytes. Evidence of granularity and cell death becomes apparent as early as 24 hours after infection with both MHV-3 and MHV-4 (Panels A and E). No differential effect was observed in the pattern of cytopathic effects on hepatocytes at later time points (48, 72, 96 hours) after infection with MHV-3 or MHV-4 (Compare panels B and F, C and G, D and H).

Comparison of MHV-3 and MHV-4 Effects on Brain Endothelial Cells

Fig. 3 compares the cytopathic effects on brain endothelial cells at 24, 48, 72 and 96 hours after infection with MHV-3 or MHV-4. In contrast to the findings obtained with hepatic endothelial cells treatment with the hepatotropic MHV-3 did not result in any cytopathic effects on brain endothelial cells at 24 hours (Panel A). The earliest evidence of cytopathology was at 96 hours after treatment of brain endothelial cells with MHV-3 (Panel D). Treatment of brain endothelial cells with the neurotropic MHV-4 resulted in extensive cellular destruction by 72 hours following infection (Panel G).

Two key finding are of interest. They are 1. Brain endothelial cells are not readily susceptible to the hepatotropic MHV-3 when compared to hepatic endothelial cells and 2. Brain endothelial cells are more susceptible to the neurotropic MHV-4 infection, as evidenced by the time course of cytopathic effects, when compared to the hepatotropic MHV-3.

Comparison of MHV-3 and MHV-4 Titers in Supernatants of Hepatic Endothelial Cells and Hepatocytes

The data presented in Table I demonstrate that the hepatotropic MHV-3 readily replicates in hepatic endothelial cells with viral titers peaking at 48 hours following infection (3.2×10^5 PFU/ml). In contrast, the hepatic endothelial cells did not support the replication of the neurotropic MHV-4 at any of the time points examined (0 PFU/ml at all time points). The selective ability to support MHV-3 replication was found to be restricted only to the endothelium. Hepatocytes were able to support the replication of both MHV-3 and MHV-4, with equivalent viral titers obtained at 96 hours after infection ($1.9\text{-}2 \times 10^5$ PFU/ml)(Table II).

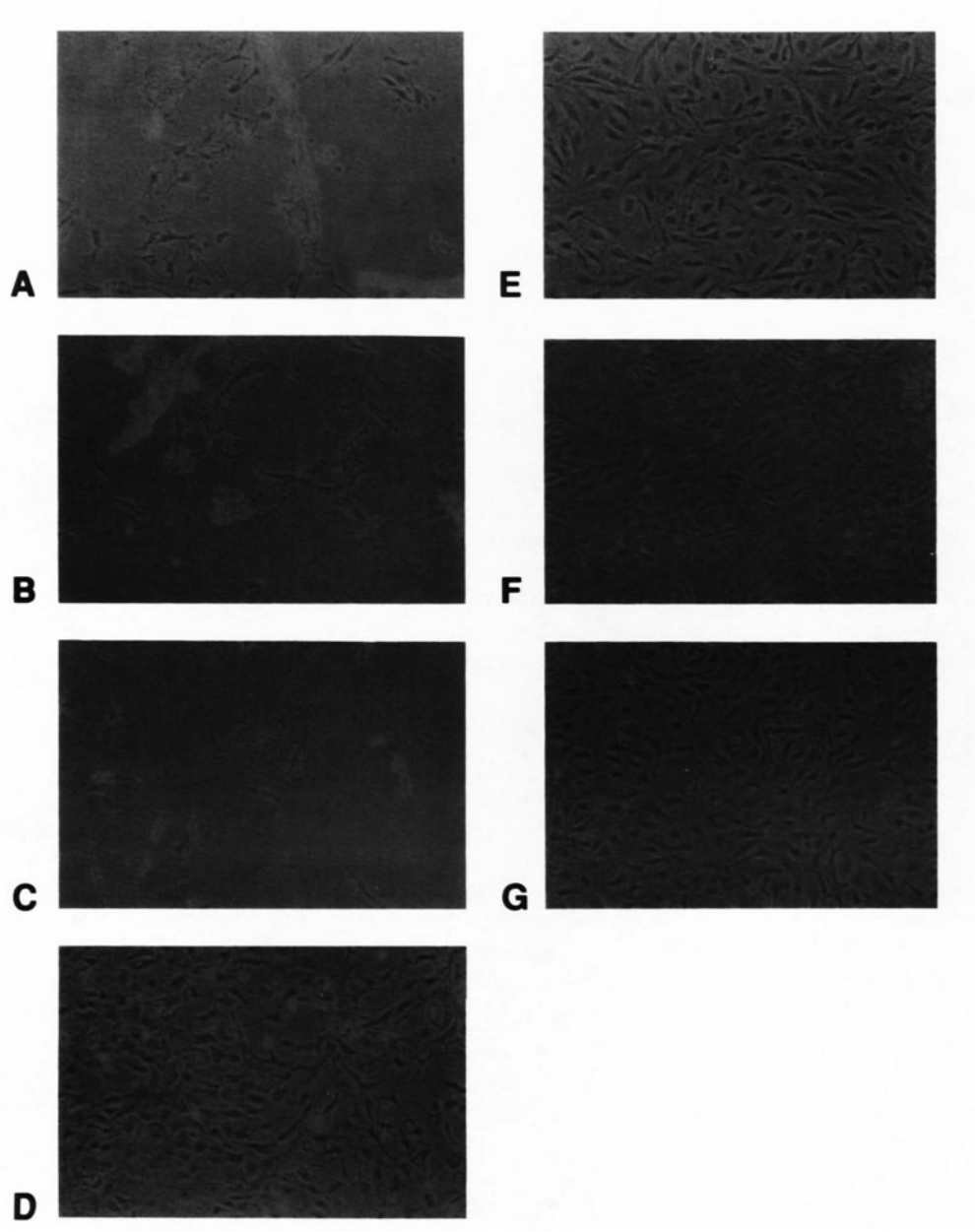

Figure 1. MHV-3 and MHV-4 induced cytopathology following infection of hepatic endothelial cells at an MOI of 0.1. Panels A. MHV-3, 24hrs, B. MHV-3, 48 hrs, C. MHV-3, 72 hrs, E. MHV-4, 24 hrs, F. MHV-4, 48 hrs, G. MHV-4, 72 hrs, D. MHV-4, 96 hrs. Syncytium formation as well as rapid cellular destruction occurs as early as 24 hours after infection with MHV-3. The cells remaining relatively intact following MHV-4 treatment as late as 96 hours after infection.

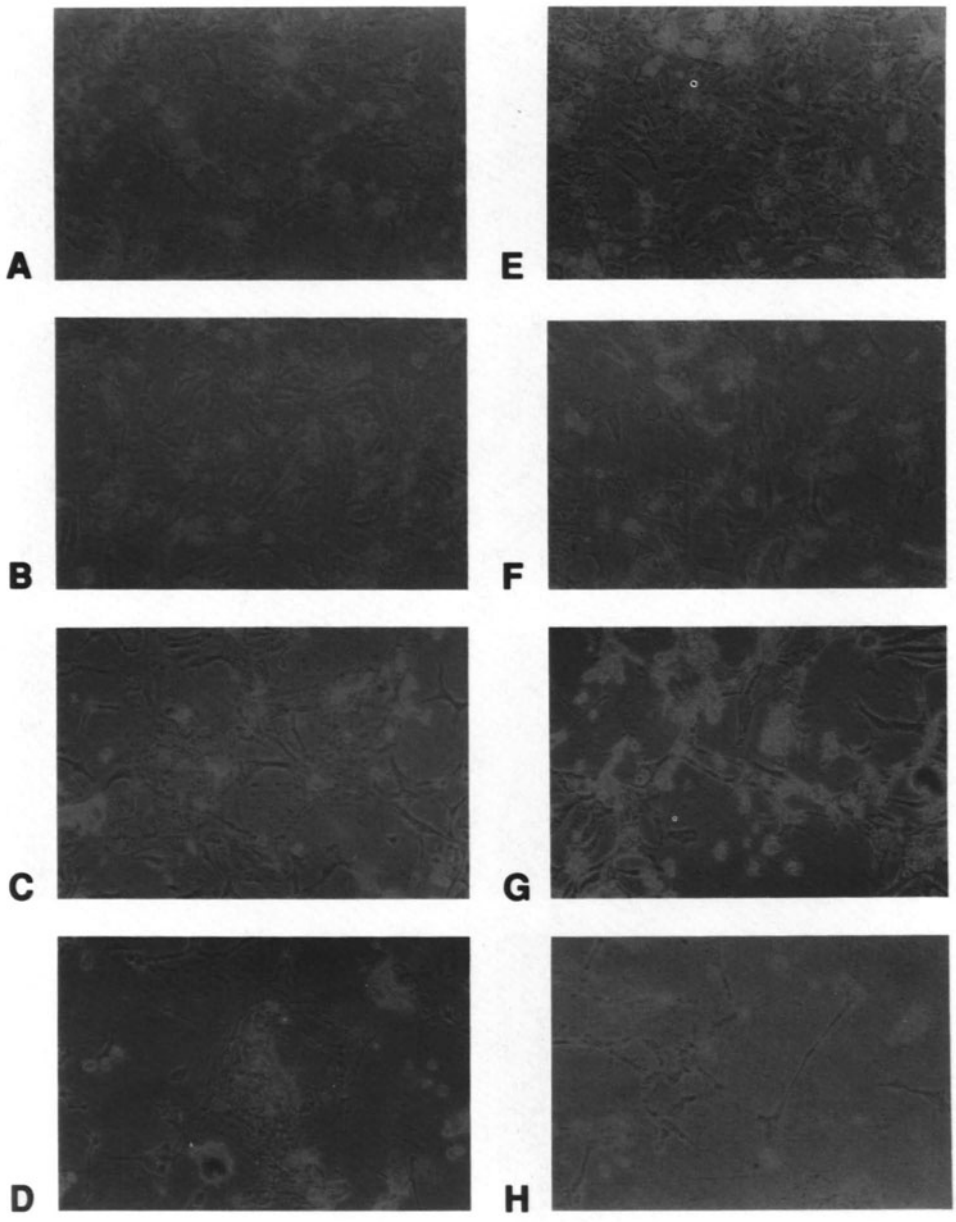

Figure 2. MHV-3 and MHV-4 induced cytopathology following infection of hepatocytes. Panels A. MHV-3, 24 hrs, B. MHV-3, 48 hrs, C. MHV-3, 72 hrs, D. MHV-3, 96 hrs, E. MHV-4, 24 hrs, F. MHV-4, 48 hrs, G. MHV-4, 72 hrs, H. MHV-4, 96 hrs. Comparable cytopathology is induced by both virus strains. Granularity and cell death starts at 24 hours with gradual progression to total destruction of the monolayer by 96 hours.

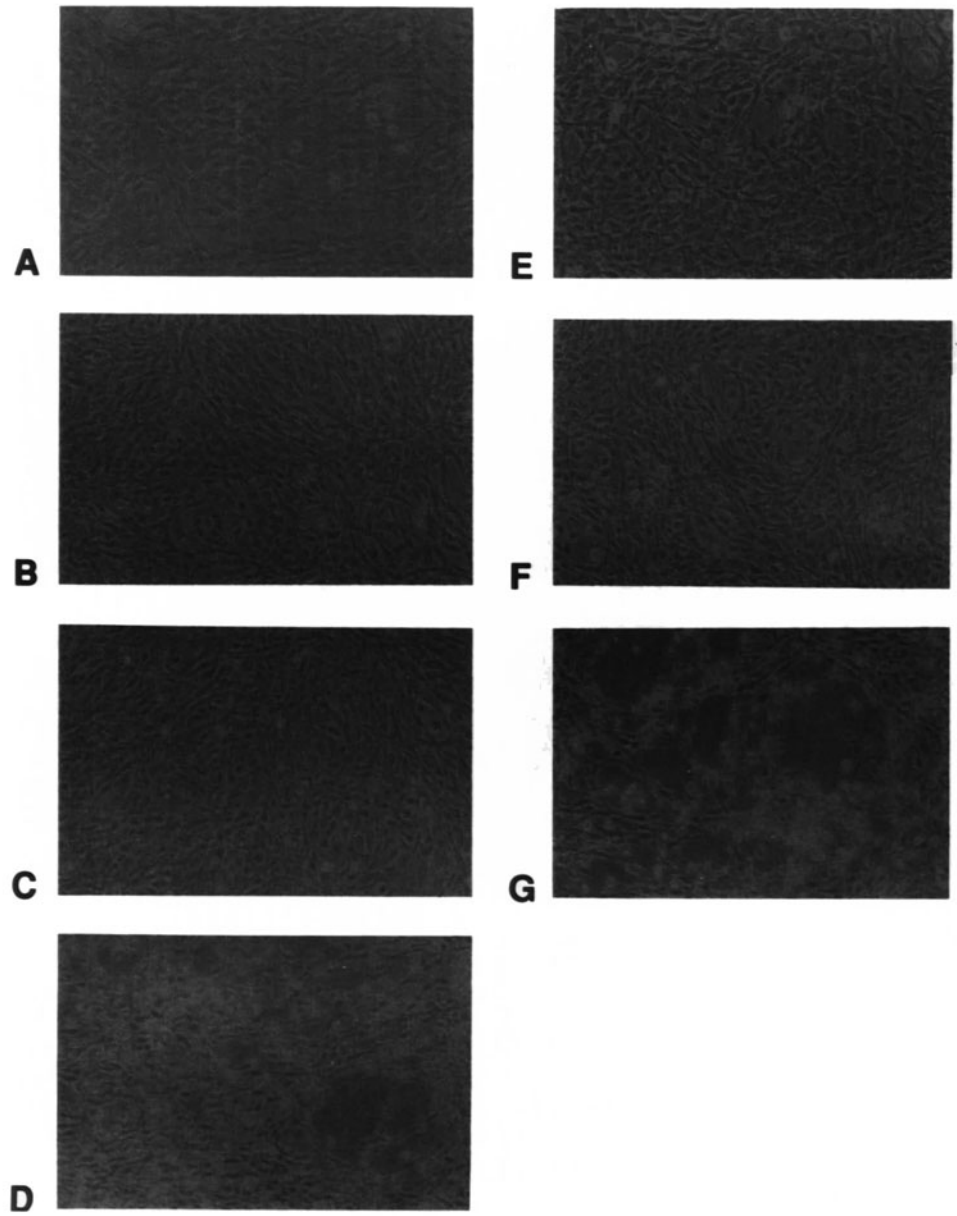

Figure 3. MHV-3 and MHV-4 induced cytopathology following infection of brain endothelial cells. Panels A. MHV-3, 24 hrs, B. MHV-3, 48 hrs, C. MHV-3, 72 hrs, D. MHV-3, 96 hrs, E. MHV-4, 24 hrs, F. MHV-4, 48 hrs, G. MHV-4, 72 hrs. MHV-4 is more cytopathic on these cells with total cellular destruction occuring by 72 hours while it takes up to 96 hours for this to occur with MHV-3.

DISCUSSION

The selective organ tropism of different strains of MHV are regulated by multiple factors such as age, dose, route of administration, and strain of experimental animals[1]. Additional factors that regulate MHV tropism are likely to be involved, depending on the

Table I. Comparison of neurotropic (MHV-4) and hepatotropic (MHV-3) virus replication in hepatic endothelium

Time (h)	MHV-4 (PFU/ML)	MHV-3 (PFU/ML)
0	0	0
24	0	2.9×10^5
48	0	3.2×10^5
72	0	4×10^4
96	0	0

manner of spread of virus within the body For instance hematogenous spread of virus occurs following infection of mice through the natural intranasal route of infection[8] The hematogenous spread can be selectively regulated by the endothelial cells, that form a barrier between blood and tissue, in different organs Endothelial cells are heterogeneous in different organs of the body and express organ specific cell surface properties as well as blood-brain barrier function in the brain[2]

The goal of the current study was to determine if endothelial cells from brain and liver demonstrate any heterogeneity in its response to the predominantly neurotropic MHV-4 (JHM) and hepatotropic MHV-3 strains The results obtained demonstrate an organ specific pattern of cytopathology and virus replication in endothelial cells, which mirrors the tropism of the virus strains In sharp contrast, the hepatocytes do not discriminate between the neurotropic and hepatotropic strains of the virus Hepatocytes support equivalent replication of both MHV-4 and MHV-3 These findings point toward an important regulatory role for the endothelial cells in organ specific virus infection that could ultimately influence their tissue tropism

Several possible mechanisms are likely to be involved in regulating differential effects of MHV strains on organ specific endothelial cells One possible difference between endothelial cells may lie in the heterogeneity of virus receptor expression Such differences have been previously described for murine cocksackievirus In this model it was found that the tropism of the virus correlated with their ability to infect endothelial cells from different organs as well as virus receptor expression on these cells[4] The MHV receptor has been identified as a member of the carcinoembryonic antigen family and several isoforms have been identified that show differences in organ and strain distribution[9,10] Heterogeneity in the expression of MHV receptor isoforms on organ specific endothelial cells could influence differential virus binding and replication of the various MHV strains This possibility is an area of further study

Recent studies in other laboratories have suggested that the presence of a functional virus receptor alone may not be sufficient to confer susceptibility to MHV infection Multiple cellular factors that influence virus internalization, virus uncoating or other steps in virus

Table II. Comparison of neurotropic (MHV-4) and hepatotropic (MHV-3) virus replication in hepatocytes

Time (h)	MHV-4 (PFU/ML)	MHV-3 (PFU/ML)
0	50	0
24	3.1×10^3	0
48	2.4×10^4	4.3×10^3
72	4.4×10^5	5.6×10^4
96	2.0×10^5	1.9×10^4

replication could determine virus susceptibilty in different endothelial cell populations[11 13] The *in vitro* endothelial cell system offers an opportunity to dissect out the various parameters in organ selective infection of the vascular beds, which could provide important insights into understanding the mechanisms of MHV tissue tropisms

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