IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Cytomegalovirus Initiates Infection Selectively from High-Level β 1 Integrin—Expressing Cells in the Brain

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Cytomegalovirus (CMV) is a prevalent pathogen in intrauterine infections that causes congenital anomalies such as CMV encephalitis, which is characterized by the focal areas of reactive gliosis, reactive mononuclear cells, microglial nodules, and ventriculoencephalitis. To elucidate the mechanisms of CMV susceptibility in the developing brain, cell tropism and the infectious dynamics of CMV infection were investigated. We evaluated intraventricular and intravascular infections from the perspective of the distribution of CMV and its receptor (β 1 integrin) in the earliest phase of infection. Murine CMV (MCMV) immediate early 1—positive cells were colocalized mainly with meninges and choroid plexus (after intraventricular infection) or with endothelial cells and pericytes (after intravascular infection). Using green fluorescent protein—expressing recombinant MCMV particles and fluorescent microbeads (100 to 300 nm), we revealed that CMV particle size is the primary factor determining the initial CMV distribution. β 1 Integrin inhibition using a shRNA and functional blocking antibody significantly reduced MCMV infection. IHC analysis, flow cytometric, and brain slice analyses strongly support that high-level β 1 integrin—expressing cells (eg, endothelial cells, pericytes, meninges, choroid plexus, and neural stem progenitor cells) are the first targets of MCMV. Therefore, our data demonstrate that the initial distributions of MCMV particles and β 1 integrin determine the distinct pattern of infection in the brain in the acute phase. (*Am J Pathol* 2015, 185: 1304–1323; <http://dx.doi.org/10.1016/j.ajpath.2015.01.032>)

Cytomegalovirus (CMV) is a member of the herpesvirus family and is one of the most important infectious pathogens of intrauterine infections causing congenital anomalies. Intrauterine infection with human CMV (HCMV) causes a variety of congenital abnormalities, depending on the timing of infection, infectious route, and virulence of the virus.¹ Of 4 million annual births, approximately 40,000 children (0.2% to 2% of all deliveries) are born with CMV, resulting in approximately 400 fatal cases each year in the United States.² Fatally infected infants have generalized CMV inclusion disease at birth, which is characterized by symptoms such as microcephaly, periventricular calcification, cerebellar hypoplasia, and ocular abnormalities (eg, microphthalmia and optic nerve atrophy).^{3,4} Nearly 10% of nonfatally infected infants

exhibit subclinical congenital infection but subsequently develop brain disorders, such as mental retardation, sensorineural hearing loss, visual defects, seizure, and epilepsy.^{5,6}

Histopathological findings from the brains of autopsied infants with CMV encephalitis include the focal areas of reactive mononuclear cells, reactive gliosis, microglial nodules, and ventriculoencephalitis characterized by inflammation and necrosis involving ependymal and subependymal cells.^{4,7} Brain microvascular endothelial cells (ECs), astrocytes, neuronal cells, oligodendroglial cells, macrophages/microglia, and neural stem/progenitor cells (NSPCs) are all

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susceptible to CMV infection.^{4,8} However, these different cell types vary in their ability to support a complete viral replication cycle, which in turn is largely controlled by the transcription factors within the cell during infection. The unique pattern of CMV infection of the brain is considered highly dependent on appropriate cellular transcription factors that bind to the DNA sequence of the CMV immediate early (IE) enhancer/promoter in the developing brain.^{9,10} How CMV particles disseminate in the brain during the earliest phase of infection is unknown.

In utero infection is believed to be a result of maternal viremia followed by hematogenous spread to the fetus from the placenta. There are two main infectious routes to the brain. The first is the hematogenous route, which involves infection of the ECs of the brain. The second is the intraventricular route. Once within central nervous system (CNS), viruses may spread through the subarachnoid space, meninges, and choroid plexus via cerebrospinal fluid.¹¹ The penetration of CMV particles through the blood–brain barrier (BBB) allows them to reach the parenchyma of the neonatal brain. The function of the BBB in the congenital and neonatal phase is controversial, and reports on the disruption of BBB during congenital CMV infection are few. The BBB acts as an interface between the peripheral circulation and CNS, allowing nutrients into the CNS but preventing blood-borne pathogens from harming the brain.^{12,13} The BBB is formed by capillary ECs connected by tight junctions (TJs). TJs of the capillary endothelium are supported by astrocytic endfeet and pericytes. CNS vessels have the highest pericyte coverage of any vessels, and the extent of pericyte coverage in vessels throughout the body inversely correlates with the relative permeability of these vessels.^{14,15} Alcendor et al¹⁶ found evidence of HCMV infection of pericytes using dual-label immunohistochemistry (IHC) analysis, as monitored by NG2 proteoglycan staining.

Intraventricular inoculation with murine CMV (MCMV) is used to establish CNS infection in mice. Studies using this technique report widespread infection, particularly of cells in the periventricular zones and in regions of the brain in direct contact with cerebrospinal fluid, similar to HCMV ventriculoencephalitis.⁷ Previously, we analyzed the effects of CMV infection on brain development by intraventricular infection,¹⁷ placental infection,¹⁸ and i.p. infection^{9,19} focusing mainly on ventricular infection. The viral infection inhibits proliferation and differentiation of the NSPCs to neuronal and glial cells in addition to induction of neuronal cell loss. These cellular events may cause brain malformations, such as microcephaly and polymicrogyria.²⁰ However, in these models, it was difficult to investigate the effects of MCMV infection on cerebral corticogenesis in terms of infection and the stage of CNS development because we mainly focused on CMV ventricular infection. On the other hand, Koontz et al²¹ developed a mouse model of HCMV infection of the developing CNS of hematogenous origin using peripheral i.p. inoculation of newborn mice with low levels of MCMV.

Infected cells were detected in different regions of the brain, including the frontal cortex, hippocampus, and cerebellum. There have been no precise pathological analyses from the perspective of primary virus distribution for congenital CMV infection. In this study, we performed a direct injection of MCMV into both the cerebroventricular zone and blood vessels to investigate the neonatal brain infection at the earliest phase of infection. At present, no vaccine is available against congenital CMV infection. It is extremely important to analyze the primary infection mechanism to prevent CMV infection in the CNS.

HCMV/MCMV initiates infection through a nonspecific, low-avidity interaction with heparan sulfate proteoglycans (HSPGs).²² Cellular integrins function as entry receptors for CMV. $\beta 1$ Integrin has been shown to interact with HCMV/MCMV particles and trigger intracellular signaling through a disintegrin-like motif of the envelope glycoprotein B.^{23,24} In MCMV infection, the only identified entry receptor is $\beta 1$ integrin.²³ To the best of our knowledge, the distribution of receptors for CMV has not been studied in the brain. We evaluated intraventricular infection and intravascular infection *in vivo* to investigate the relationship between the level of expression of $\beta 1$ integrins and the distribution of MCMV particles in the brain during the first viremic phase. Our data demonstrate that the initial distributions of MCMV particles and $\beta 1$ integrin-expressing cells determine the distinct pattern of infection in the brain in the acute infection phase.

Materials and Methods

Mice

ICR mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The day of birth was designated as postnatal day 0 (P0). All experiments were approved by, and conducted according to the guidelines of, the Institutional Animal Care and Use Committee of the Hamamatsu University School of Medicine (Hamamatsu, Japan). All efforts were made to minimize both the number of animals used and their suffering.

Viruses and the Generation of Recombinant M32-EGFP-MCMV

The Smith strain of MCMV was passaged in mouse embryonic fibroblasts. Mouse embryonic fibroblasts were prepared from 12-day-old ICR mouse embryos, as previously reported,²⁵ and grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 100 U penicillin, 50 mg/mL streptomycin, and 10% fetal calf serum. The virus was quantified by the plaque assay method. The concentration of the virus stock was 1×10^8 plaque-forming units (PFU)/mL. Recombinant viruses, derived from the Smith strain of wild-type MCMV (<http://www.ncbi.nlm.nih.gov/nucleotide>; Accession number U68299) and capable of expressing the enhanced green fluorescent protein (EGFP) reporter (Clontech Laboratories Inc., Palo Alto, CA) were used in this study.

EGFP was inserted between 37,089 and 41,450 (4361 Bp; the M32–M31 locus) in the MCMV genome by homologous recombination. The MCMV M32 (41,450 to 39,286; left flank) and M31 (37,089 to 39,246; right flank) gene loci were amplified by conventional PCR method using the following primers: M32: forward, 5'-CTACTAGCTAGCCTTCCGCGAGTCGCTGTATT-3', reverse, 5'-CTACTAGCTAGCCTTCCGCGAGTCGCTGTATT-3'; and M31: forward, 5'-CTAAATTAAGTTCGTCCTCTTCGTCTCACA-3', reverse, 5'-AACTAGTCTAGATCGCTCCTGGTTGGTTTTTA-3'.

The M32 and M31 gene loci were then inserted into the pEGFP-N1 vector (Clontech) flanking the EGFP gene locus. The M32-EGFP-M31 gene locus was cleaved with *NheI* and *AflIII* from the M32-EGFP-M31 recombination plasmid and dissolved in water. The cleaved construct was transfected into the nuclei of MCMV (Smith strain)-infected NIH3T3 cells 24 hours postinfection (hpi) using an electroporation system (Amaxa Biosystems, Hamburg, Germany) to induce homologous recombination. At 3 days after infection, cells were co-cultured with uninfected mouse embryonic fibroblasts at a ratio of 1:1000 in 6-well plates and screened for EGFP-expressing foci of infected cells. Virus-containing supernatant from wells containing green fluorescent foci was harvested and diluted 10-fold. Wells that displayed single green fluorescent foci were chosen for purification. A virus preparation was considered pure when all foci resulting from limiting-dilution infection with the preparation displayed EGFP expression, indicating that no wild-type virus remained. The virus was quantified by the plaque assay method. The concentration of the virus stock was approximately 1×10^8 PFU/mL. Previously generated human elongation factor (hEF)-EGFP–recombinant MCMV (1×10^8 PFU/mL) was used in this experiment.²⁶

Gradient Purification of MCMV Virions

The gradient purification of MCMV (Smith strain) and M32-EGFP-MCMV virions was performed according to the method of Britt.²⁷ Infectious supernatants from infected mouse embryonic fibroblast cultures were made cell free by centrifugation at $3000 \times g$ for 20 minutes at 16°C. The supernatants were then ultracentrifuged for 40 minutes at $70,000 \times g$. Pellets containing virions and other particles were resuspended in 1 mL of Tris-buffered saline and transferred onto a preformed linear sorbitol gradient (25% to 70%), which was ultracentrifuged at $70,000 \times g$ for 60 minutes. The virion-containing band was harvested with a syringe and the virions were washed and pelleted by additional ultracentrifugation at $70,000 \times g$ for 40 minutes. The pellet was resuspended in phosphate-buffered saline (PBS) and stored at –80°C until infection experiments.

Intraventricular Injection of MCMV into Neonatal Mice

Normal ICR mice were purchased from Japan SLC, Inc. and maintained in our animal center. On their day of birth,

(designated as P0.5), 5 μ L of MCMV (approximately 5×10^5 PFU) was injected into the lateral ventricle at the midpoint between the ear and eye using a 10- μ L Hamilton syringe with a 27-gauge needle. The solution contained 0.05% (w/v) trypan blue to help determine whether the ventricles were successfully injected. Brains were harvested at a range of time points postinjection.²⁸

I.V. Injection of MCMV into Neonatal Mice

A 1-mL syringe with a 35-gauge needle was used to perform the i.v. injection of MCMV. A Wee Sight Transilluminator (Phillips Healthcare, Andover, MA) was used to visualize the superficial temporal (facial) vein. We slowly infused 5×10^6 PFU (50 μ L) of MCMV into the superficial temporal vein of neonatal mice. For the mannitol study, 0.3 mL of sterile 25% mannitol in 0.9% saline per 10 g body weight was injected i.p. 20 minutes before the i.v. injection of MCMV, as previously described.²⁹

Near-Infrared Fluorescent Microbeads

Carboxyl fluorescent Nile red particles 0.04 to 0.06 μ m, 0.1 to 0.3 μ m, and 1.7 to 2.2 μ m in size were purchased from Spherotech Inc. (Lake Forest, IL). The maximum excitation and emission wavelength of these beads are 485 and 525 nm, respectively. The beads were treated with 0.05 mol/L NaOH for approximately 1 day to remove any endotoxins and resuspended in sterile water. Before use, the beads were adsorbed overnight with 10% mouse serum prepared from C57BL/6 mice. To separate aggregates, the beads were vortexed and sonicated thoroughly before use. Approximately 50- μ L beads (0.04 to 0.06 μ m, approximately 3.63×10^{11} particles; 0.1 to 0.3 μ m, approximately 5.75×10^9 particles; and 1.7 to 2.2 μ m, approximately 6.85×10^6 particles) were injected i.v. into mice and 5 μ L (0.1 to 0.3 μ m, approximately 5.75×10^8 particles) were intracranially injected into mice.

Immunofluorescence Staining

Snap-frozen fresh tissues in precooled n-hexane (–80°C) were embedded in optimal cutting temperature compound in cryomolds. Frozen blocks were stored at –80°C until use. Cryostat sections 8 to 10 μ m thick were cut and mounted on Superfrost Plus slides (Thermo Fisher Scientific, Rockford, IL). The samples were immediately fixed with Cytokeep II (Nippon Shoji Co., Osaka, Japan), air-dried for 30 minutes, and stored at –80°C until use. The sections were incubated in a blocking solution (10% goat serum in PBS) for 10 minutes at room temperature, incubated with an unconjugated primary antibody for 30 minutes at room temperature, and washed in PBS. The sections were then incubated with an Alexa Fluor–conjugated secondary antibody (Molecular Probes; Life Technologies Corp., Carlsbad, CA) or stained with fluorescein isothiocyanate–conjugated *Griffonia simplicifolia* isolectin B4 (Vector laboratories, Inc., Burlingame, CA). The sections were

mounted in ProLong Gold (Molecular Probes; Invitrogen, Carlsbad, CA) and imaged with a fluorescence microscope (Keyence, Tokyo, Japan).

IHC Analysis

Brains were fixed in 10% buffered formalin, embedded in paraffin, and sectioned to yield slices 4 μ m thick. After the sections were deparaffinized, they were initially incubated in 3% hydrogen peroxide solution for 10 minutes at room temperature. Antigen retrieval was performed according to the manufacturer's instructions. The sections were incubated with primary antibodies, such as MCMV IE1, β 1 integrin, CD31, and matrix metalloproteinase (MMP)-9, in blocking solution (10% goat or rabbit serum and 0.1% Triton X-100 in PBS) for 30 minutes at room temperature or overnight at 4°C. After washing in PBS, the sections were incubated with peroxidase-conjugated universal immunoenzyme polymer anti-mouse, anti-rabbit, and anti-goat solution (Nichirei Biosciences, Tokyo, Japan), and visualized using 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). IHC double-staining was also performed. Sections were first reacted with antibodies against MCMV IE1 and nestin, incubated with biotinylated goat anti-mouse and anti-rabbit antibodies and alkaline phosphatase-conjugated streptavidin, and then colored with Fast Blue BB Salt (Sigma-Aldrich) in the presence of naphthol AS-MX phosphate (Sigma-Aldrich).

Antibodies

The following primary antibodies were used: mouse anti-MCMV IE1 (IgG1, 1:1000; Croma 101; kindly gifted by Stipan Jonjic, University of Rijeka, Rijeka, Croatia); fluorescein isothiocyanate- or phosphatidylethanolamine (PE)-conjugated rat anti-CD31 (1:100; eBioscience, Inc., San Diego, CA); rabbit anti-CD31 (1:100; Abcam, Cambridge, UK); PE-conjugated Armenian Hamster anti- β 1 integrin (anti-CD29) (1:100; eBioscience); fluorescein isothiocyanate-conjugated rat anti-PDGFR β (1:100; eBioscience); rabbit anti-NG2 (1:100; EMD Millipore Inc., Billerica, MA); rabbit anti-nestin antiserum (1:100; kindly gifted by Dr. Hiroshi Kitani, National Institute of Animal Health, Tsukuba, Japan)³⁰; rat anti-F4/80 (1:100), which is specific for mouse macrophages/microglia (AbD Serotec, Kidlington, UK); rabbit anti-glial fibrillary acidic protein (GFAP; Dako Japan Inc., Tokyo, Japan); rabbit anti-oligodendrocyte transcription factor Olig2 (1:100; IBL Co., Ltd., Gunma, Japan); rabbit anti-microtubule-associated protein 2 (1:100; Abcam); mouse anti-HS antibody 10E4 (1:100; Seikagaku Corp., Tokyo, Japan); rabbit anti-epidermal growth factor receptor (EGFR) (1:50; Cell Signaling Technology, Beverly, MA); rabbit anti-PDGFR α (1:1000; Cell Signaling); goat anti-MMP-9 (1:200; R&D Systems Inc., Minneapolis, MN); and rabbit anti-claudin-5 (1:100; Invitrogen). The second antibodies used were: Alexa Fluor 350-conjugated goat anti-mouse IgG1 (Molecular Probes); Alexa Fluor

488-conjugated goat anti-mouse IgG1 (Molecular Probes); Alexa Fluor 546-conjugated goat anti-mouse IgG1 (Molecular Probes); Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes); Alexa Fluor 546-conjugated goat anti-mouse IgG (Molecular Probes); Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes); Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes); peroxidase-conjugated goat anti-mouse IgG (Nichirei Biosciences); and peroxidase-conjugated goat anti-rabbit IgG (Nichirei Biosciences).

In Situ Hybridization

The probe for DNA *in situ* hybridization (ISH) was made from the MCMV DNA genome (*pSM3fr*) by nick translation using a bacterial artificial chromosome system. ISH with MCMV DNA was performed as previously described.²⁶ The tissue sections were treated with RNase (100 μ g/mL in PBS; Boehringer Ingelheim, Ingelheim am Rhein, Germany) for the detection of viral DNA.

Generation of Stable Knockdown Cell Lines Using a shRNA against β 1 Integrin

Stable knockdown cell lines were generated using a β 1 integrin shRNA plasmid (sc-44311-SH) and a control shRNA (sc-108060; Santa Cruz, Biotechnology, Santa Cruz, CA). Each plasmid was transfected into NIH3T3 cells with FuGENE HD Transfection Reagent (Promega Corp., Madison, WI). Forty-eight hours after transfection, the medium was aspirated and replaced with fresh medium containing 5 μ g/mL puromycin. Approximately every 2 to 3 days, the medium was aspirated and replaced with freshly prepared selective media. Selected clones were analyzed by flow cytometry using an antibody against β 1 integrin.

Western Blot Analysis

MMP-9 and claudin-5 were detected using a goat antibody specific to human and mouse MMP-9 (R&D), and a rabbit polyclonal antibody specific to claudin-5 (Invitrogen). Sample loads were standardized against β -actin using a mouse monoclonal antibody specific to β -actin (Sigma-Aldrich). The blots were incubated with a biotin-conjugated secondary antibody (Nichirei) followed by the horseradish peroxidase-conjugated avidin-biotin reaction. Immunoreactive bands were visualized using an enhanced chemiluminescence substrate (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Flow Cytometry

Flow cytometry was performed according to the method of Panchision et al.,³¹ with some modifications. Brains were infected with hEF-EGFP-MCMV by i.v. or intraventricular injection. Infected brains were harvested and samples were centrifuged at 200 $\times$ g for 5 minutes, resuspended in fresh Hank's balanced salt solution/DNase/magnesium chloride

without enzyme, and triturated with a pipette tip three times. The tissue was allowed to settle for 5 minutes, and the top 800 μL of suspended cells were transferred into a new tube to avoid further disturbance. The combined cell suspensions of each group (ie, intraventricular infection, i.v. infection without mannitol, and i.v. infection with mannitol), were centrifuged and resuspended in medium appropriate for further use. The cells were subjected to 60 minutes incubation with 200 $\mu\text{g}/\text{mL}$ Liberase DH (Roche Diagnostics, Mannheim, Germany), corresponding to 0.62 Wünsch units/mL collagenase and 66.7 Wünsch units/mL dispase. After filtration through a nylon screen (BD Biosciences, San Diego, CA) to remove aggregates, the cells were centrifuged and rinsed twice in fluorescence-activated cell sorting (FACS) buffer [1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 2% heat-inactivated fetal calf serum, 120 $\mu\text{g}/\text{mL}$ penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin in Hank's balanced salt solution]. First, whole isolated cells were incubated with allophycocyanin-conjugated anti-CD31 antibody ($\times 100$), PE-conjugated PDGFR β antibody ($\times 100$), allophycocyanin-conjugated anti-PDGFR α antibody ($\times 100$), anti-PE-Cy7-conjugated anti-CD29 antibody ($\times 100$), and rabbit anti-EGFR antibody for 30 minutes on ice. After centrifugation and rinsing twice in FACS buffer, some cells were incubated with anti-rabbit Alexa Fluor 350 ($\times 200$) for 30 minutes on ice. After centrifugation and rinsing twice in FACS buffer, sorting and analysis were performed using a FACS Aria (BD Biosciences). We sorted the GFP-positive infected cells from whole isolated cells and examined the ratio of each CD31-, PDGFR β -, PDGFR α -, CD29-, and EGFR-positive cells, respectively among GFP-positive cells by FACS.

Cell/Particle Counts in Tissue Sections

The numbers of EGFP-positive MCMV particles and Nile red-positive microbeads were counted in coronal frozen sections of brains. The numbers of cells double-positive for MCMV IE1 and CD31, PDGFR β , NG2, GFAP, F4/80, Olig2, or microtubule-associated protein 2 were counted in coronal frozen sections of MCMV-infected brains. The numbers of particles or double-positive cells were counted in the meninges/choroid plexus and parenchyma of the brain independently.

Preparation of Cerebral Slices

Brain tissue was obtained from 0- and 21-day-old ICR mice. Cerebral slice culture was performed according to the method of Kawasaki et al.³² Whole brains, dissected from each mouse and kept in cold Hank's balanced salt solution (Gibco BRL, Grand Island, NY) on ice, were fixed on a stage with instant glue and cut into coronal sections 200 μm thick with a microslicer (Dohan EM, Kyoto, Japan). Slices were transferred to porous transparent membranes (Millicell CM, 0.4 mm; Millipore) that were floated on culture medium in a 6-well plate (Becton, Dickinson, and Co., Franklin Lakes, NJ), as previously reported.³² The medium consisted of

Neurobasal-A medium (Gibco BRL) supplemented with B27 (Gibco BRL), 0.15 mg/mL glutamine, 100 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. The whole brain slices were immersed in hEF-EGFP-MCMV for 1 hour at 37°C and washed three times with PBS. The slices were maintained at 37°C in a humidified incubator with 95% air and 5% CO₂.

Matrix Metalloproteinase Activity Assay

A SensoLyte 520 Generic MMP assay kit (AnaSpec Inc., Fremont, CA) was used to detect the activity of MMP 1 to 3, 7 to 9, and 12 to 14. The kit uses 5-carboxyfluorescein-(fluorophore)— and QXL520 (quencher)-labeled Förster resonance energy transfer peptide substrates for continuous measurement of enzyme activities. In an intact Förster resonance energy transfer peptide, the fluorescence of 5-carboxyfluorescein is quenched by SensoLyte; however, on the cleavage of the Förster resonance energy transfer peptide by MMPs, the fluorescence of 5-carboxyfluorescein is recovered and can be continuously monitored at excitation and emission wavelengths of 490 and 520 nm, respectively. The assays were performed in a 96-well plate.

Functional Blocking with Anti- $\beta 1$ Integrin Antibody *in Vitro* and *in Vivo*

Cells, brain slices, and brains *in vivo* were treated with purified Armenian hamster functional blocking anti- $\beta 1$ integrin antibody (clone Ha2/5: BD Biosciences) or purified Armenian Hamster IgM isotype control antibody (BD Bioscience) before MCMV infection. NIH3T3 cells were treated with antibodies at appropriate concentrations (0, 12.5, 25, and 50 $\mu\text{g}/\text{mL}$) for 30 minutes and incubated with recombinant MCMV (hEF-EGFP-MCMV) at multiplicity of infection of 0.5. After 24 hpi, infected cells were observed and the ratios of infected cells were examined by using FCM. Brain slices on membranes were covered with functional blocking anti- $\beta 1$ integrin antibody (20 $\mu\text{g}/\text{mL}$) or isotype control antibody (20 $\mu\text{g}/\text{mL}$) for 1 hour and infected with recombinant virus (hEF-EGFP-MCMV) (5×10^6 PFU/mL) over their entire surfaces. They were incubated for 1 hour with occasional shaking, washed two times with Hank's balanced salt solution, and cultured in the slice culture medium as described in the previous section. At 24 hpi, brain slices were fixed with paraformaldehyde and infected cells were counted. Neonatal mice (P0) were injected with functional blocking anti- $\beta 1$ integrin antibody (50 μL , 20 $\mu\text{g}/\text{g}$) or isotype control antibody (50 μL , 20 $\mu\text{g}/\text{g}$) into the superficial temporal vein. One hour after antibody injection, 5×10^6 PFU (50 μL) of MCMV (Smith strain) were infused into the other side of the temporal vein. At 24 hpi, infected brains were removed and fixed with 4% paraformaldehyde. The paraffin sections were prepared and immunostained with antibody against MCMV IE1. MCMV IE1-positive cells in the two coronal sections (anterior side section and posterior side section) of each brain were counted, and the numbers

were compared between the functional blocking group ($n = 5$) and the control group ($n = 5$).

Results

Intraventricular Infection with MCMV

MCMV (5 μ L, 5×10^5 PFU) was injected into the brains of neonatal mice (ICR) by intraventricular injection as described in *Materials and Methods*. The proper injection of one ventricle allowed the distribution of trypan blue from the injected side to the opposite side of the brain. The uniform distribution of trypan blue in both the right and left cerebral hemispheres was visible approximately 60 minutes after injection (*Supplemental Figure S1*). It is unclear how CMV particles disseminate throughout the brain after intraventricular delivery; we hypothesized that MCMV particles spread immediately through the brain like trypan blue. Infected brains were harvested at a range of time points (3, 6, 12, 24, 48, and 72 hours postinfection; *Figure 1*, A–F). After formalin fixation, the expression of MCMV IE1 antigen was analyzed by IHC analysis. MCMV IE1 antigen was visible as early as 3 hpi in the choroid plexus (*Figure 1*, A and G) and marginal area (MA), including the meninges (*Figure 1*, A and H). The number of cells expressing MCMV IE1 antigen began to increase in the choroid plexus, subventricular zone (SVZ), and MA from 24 hpi. They increased in number in a time-dependent manner and had spread toward the cortex and hippocampus at 72 hpi (*Figure 1F* and *Supplemental Figure S2*).

Detection of the Murine CMV Genome by *in Situ* Hybridization in the Neonatal Brain after Its Intracerebroventricular Injection

Next, we investigated the distribution of MCMV genome in brains infected by intraventricular injection. At 3 hpi, the MCMV genome was predominantly detected in SVZ and MA (*Figure 2A*) and, to a lesser extent, in the cortex (*Figure 2B*). At 24 and 72 hpi, the MCMV genome signal increased in SVZ and MA in a time-dependent manner. Nuclei showing fused EGFP signals represent the proliferation of MCMV DNA. Compared with SVZ (*Figure 2*, D and G) and MA (data not shown), the MCMV genome signal in the cortex area (*Figure 2*, E and H) was much lower. The MCMV genome signal in the cortex gradually increased with the increasing infection time (*Figure 2*, B, E, and H). Schematic representations of the distribution of MCMV genome at 3, 24, and 72 hpi are shown (*Figure 2*, C, F, and I, respectively). These data suggest that MCMV virus particles stay in SVZ and MA immediately after injection.

MCMV Particle Distribution in the Neonatal Brain after Its Intracerebroventricular Injection

MCMV has a relatively large diameter (150 to 300 nm; *Figure 2L*). To investigate the distribution of virus particles,

Figure 1 Intracerebroventricular injection of murine cytomegalovirus (MCMV) into neonatal mice. MCMV was injected into the intracerebroventricular area of the brain. A–F: MCMV immediate early (IE) 1 expression (brown) in the brain at 3 (A), 6 (B), 12 (C), 24 (D), 48 (E), and 72 (F) hours postinfection (hpi). G and H: MCMV IE1 antigen (brown) is present as early as 3 hpi in the choroid plexus (G) and marginal area, including the meninges (H). Scale bars: 930 μ m (A–F); 310 μ m (G and H).

recombinant MCMV (M32-EGFP-MCMV) was produced as described in *Materials and Methods*. The EGFP gene was inserted between 37,089 and 41,450 (4361 Bp) in the MCMV genome by homologous recombination (*Figure 2J*). Ultracentrifuged M32-EGFP-MCMV particles were visible as green spots on fluorescence microscopy (*Figure 2K*). We confirmed that M32-EGFP-MCMV particles possess a typical virus structure using transmission electron microscopy (*Figure 2L*).

We administered 5 μ L of M32-EGFP-MCMV (5×10^5 PFU) into neonatal mouse brains (P0) by intraventricular injection. After frozen sections were made at 2 hpi, EGFP (MCMV particles) was evident mainly in SVZ (*Figure 2M*) and MA (*Figure 2N*), showing a pattern similar to that of the expression of MCMV IE1 and the distribution of the MCMV genome. We hypothesized that the size of the virus is an important factor determining the distribution pattern of the virus in the brain. We administered 5 μ L (approximately 5.75×10^8 particles) of 0.1 to 0.3- μ m fluorescent Nile red microbeads (approximately the same size as CMV) into the neonatal mouse brain by intraventricular injection. At 2

hours after injection, Nile red signals were observed in SVZ (Figure 2O) and MA (Figure 2P) in frozen sections. Therefore, it is likely that, after intraventricular injection, MCMV particles do not immediately spread uniformly throughout the parenchyma. Rather, they stay in SVZ and MA for a while and enter their cells to proliferate. On the basis of the distribution of fluorescent microbeads, the size of MCMV particles is the primary factor affecting their distribution in the brain in the acute phase of infection after intraventricular injection.

Acute Infection Phase in the Neonatal Mouse Brain after I.V. Infection with MCMV

CMV encephalitis often causes the focal areas of reactive gliosis, reactive mononuclear cells, microglial nodules, and ventriculoencephalitis. To investigate the difference in the pattern of infection of the brain between intraventricular and hematogenous infection, we performed direct intravascular MCMV injection in neonatal mice (P0.5). Although the i.p. infection of the neonatal mouse brain and i.v. infection of the adult mouse brain with MCMV have been described,^{21,33} hematogenic infection of the neonatal mouse brain has not been reported.

We injected 5×10^6 PFU of MCMV (50 μ L) into the superficial temporal vein of neonatal mice (Figure 3, A and B). At 12 hpi, MCMV IE1 antigen-positive cells were diffusely observed in the meninges and brain parenchyma (Figure 3, C and D). Most IE1-positive cells colocalized with the lectin-positive vascular area (Figure 3E). The majority of IE1-positive cells colocalized with CD31-positive ECs, but some IE1 localized in nonendothelial cells (Figure 3F). Some IE1-positive cells colocalized with PDGFR β -positive pericytes (Figure 3G).

We previously generated a recombinant virus expressing an EGFP gene insert under the control of the hEF-1 α promoter (hEF-EGFP-MCMV).²⁶ We infected neonatal mouse brains with hEF-EGFP-MCMV by i.v. injection. The isolated whole cells from the entire brain were incubated with allophycocyanin-conjugated anti-CD31 antibody and PE-conjugated anti-PDGFR β antibody for 30 minutes on ice. After centrifugation and rinsing twice in FACS buffer, sorting and analysis were performed using a FACS Aria. Flow cytometry analysis at 24 hpi showed that $6.4\% \pm 1.4\%$ of whole isolated cells were CD31-positive (ECs) and $3.0\% \pm 0.6\%$ of whole isolated cells were PDGFR β -positive

(pericyte). We sorted the GFP-positive infected cells first and examined the ratio of each double-positive cell for CD31 and PDGFR β using FACS. The mean proportion of MCMV (GFP-positive cells)/CD31 double-positive cells was $68.9\% \pm 16.4\%$. The mean proportion of MCMV/PDGFR β double-positive cells was $10.5\% \pm 2.7\%$ (Figure 3H). ISH detected the MCMV genome in the vascular area and meninges (Figure 3I). These findings indicate that MCMV particles do not diffusely spread from vessels but are initially retained by the vascular area or MA.

At 72 hpi, MCMV IE1-positive cells increased in a scattered manner, forming clusters (Figure 3J). Although the majority of MCMV IE1-positive cells were trapped in the vascular area at 12 hpi, many MCMV IE1-positive cells at 72 hpi were located outside the CD31-positive vascular area in the parenchyma (Figure 3K). In the meninges, MCMV IE1-positive cells were also located outside the CD31-positive vascular area (Figure 3L). ISH detected significantly increased MCMV genome signal compared with that shown in Figure 3I. Proliferating MCMV appeared as fused clusters with small green dots scattered around them (Figure 3, M–O). The clusters of MCMV genome signal colocalized with MCMV IE1-positive cells and were diffusely scattered around MCMV IE1-positive cells in the parenchyma (Figure 3N) and meninges (Figure 3O), indicating the spreading of MCMV infection from the primary infection foci.

Ventriculoencephalitis because of CMV has a unique, important infection pattern. SVZ was the site most susceptible to MCMV infection when brain slices from mice at different developmental stages were infected *in vitro*.³² SVZ consists mostly of undifferentiated neuroepithelial cells, which include NSPCs. NSPCs are one of the most susceptible cell types for CMV.²⁵ Nestin is an intermediate filament protein known to be an NSPC marker.³⁰ At 72 hpi, MCMV IE1-positive cells were evident in SVZ (Figure 3P), and some were double-immunostained with nestin (Figure 3Q), suggesting that MCMV reaches the SVZ area in either intravascular or intraventricular infection. The infection to SVZ including NSPCs may cause brain malformations, such as microcephaly and polymicrogyria. To observe an inflammatory response, we immunostained macrophages/microglia with anti-F4/80 antibody. F4/80-positive macrophages/microglia were prominent at the infection foci at 72 hpi. On an average, 25.2% of MCMV IE-positive cells double-stained as F4/80 positive (Figure 3R).

Figure 2 Murine cytomegalovirus (MCMV) genome distribution in the mouse brain after intracerebroventricular injection. Time course of MCMV genome distribution after intracerebrocranial infection with MCMV. The MCMV genome (green) is first detected in the subventricular zone (SVZ) (A, D, and G) and spreads to the parenchyma of the cortex (arrows, B, E, and H). C, F, and I: Scheme of the detected MCMV genome (black circles) at 3 (C), 24 (F), and 72 (I) hours postinfection (hpi). J: Enhanced green fluorescent protein (EGFP) gene was inserted between 37,089 and 41,450 (4361 bp) in the MCMV genome by homologous recombination. The M32 and M31 gene loci were inserted into the pEGFP-N1 vector flanking the EGFP gene locus by using XbaI site and BamHI site (M32-EGFP recombination plasmid). The M32-EGFP recombination plasmid M32-EGFP-M31 was cleaved with NheI and AflIII. The cleaved construct was transfected into the nuclei of MCMV (Smith strain)-infected NIH3T3 at 24 hpi for homologous recombination using an electroporation system. K: Ultracentrifuged M32-EGFP-MCMV particles are observed in green by fluorescence microscopy. L: Transmission electron microscopy reveals that M32-EGFP-MCMV particles possess a typical virus structure. M and N: EGFP dot signals (MCMV particles) are mainly observed in the SVZ (arrows, M) and marginal area (MA) (arrows, N) at 2 hpi. O and P: Nile red microbead signals (red) are observed in SVZ (O) and MA (P) at 2 hpi. Scale bars: 80 μ m (A, B, D, E, G, and H); 7.3 μ m (K); 77 nm (L); 16 μ m (M and N); 500 μ m (O); 100 μ m (P).

Mannitol Affects the Acute Infection Phase in the Neonatal Mouse Brain after I.V. Infection with MCMV

Saunders et al.³⁴ reported that the neonatal BBB is functionally effective. To understand the role of the BBB in combating CMV, we enhanced BBB disruption through i.p. mannitol treatment. Mannitol disrupts BBB by causing the osmotic shrinkage of ECs.³⁶ An i.v. injection of recombinant simian virus 40 vectors after i.p. mannitol pretreatment results in the extensive expression of multiple transgenes throughout CNS.²⁹ Mannitol treatment increased the penetration of the Evans green of the parenchyma (Figure 4A). At 12 hpi, mannitol-treated brains were harvested and IHC analysis was performed to detect the MCMV IE1 antigen. MCMV IE1-positive cells were distributed throughout the brain in a pattern similar to that of mannitol-untreated infected brains (Figures 3C and 4B). However, compared with the mannitol-untreated group (160.2 ± 29.5 cells per section), mannitol treatment significantly increased the number of MCMV IE1-positive cells at 12 hpi (337.4 ± 91.3 cells per section; $P < 0.01$) (Figure 4C). MCMV IE1-positive cells were observed in the vascular area (Lectin) of the parenchyma (Figure 4D) and meninges (data not shown). We hypothesized that mannitol treatment enhances MCMV penetration of BBB, with the result that the MCMV genome signal is ubiquitous in the brain. Unexpectedly, MCMV genome signal was found only in the vascular area (CD31-positive cells) of the parenchyma (Figure 4E) and meninges (data not shown) at 3 hpi. This finding implies that MCMV is still prevented from spreading to the parenchyma even after mannitol treatment. Mannitol treatment did not cause the complete breakage of BBB against CMV.

Identification of MCMV IE1-Positive Cells in the Acute Infection Phase after I.V. Infection

Using IHC analysis, we identified the cell types in the brain infected by MCMV during the very early phase of infection (12 hpi). Figure 4, F–Q show markers for the cells infected after mannitol treatment. Figure 4, F, J, N, H, L, P, and Q show the parenchyma and Figure 4, G, K, O, I, and M show MA. MCMV IE1-positive cells were tested for colocalization with cells positive for CD31 (endothelial marker)

(Figure 4, F and G), PDGFR β (pericyte marker) (Figure 4, H and I), NG2 (another pericyte marker) (Figure 4, J and K), GFAP (glial marker) (Figure 4, L and M), F4/80 (macrophage/microglia marker) (Figure 4, N and O) Olig2 (oligodendrocyte marker) (Figure 4P), and microtubule-associated protein 2 (neural marker) (Figure 4Q) positive cells. We also compared the proportions of infected cells expressing each marker between mannitol-treated and -untreated brains at 12 hpi by counting double-positive cells in whole sections ($n = 8$) of either the parenchyma or MA/choroid plexus areas.

In the parenchyma, the number of CD31/MCMV IE1-double-positive cells in sections of mannitol-untreated brains ($68.8\% \pm 2.9\%$) was significantly higher than that in sections of mannitol-treated brains ($54.2\% \pm 2.4\%$) ($P < 0.01$) (Figure 4, F and R). In the MA and choroid plexus, the number of CD31/MCMV IE1 double-positive cells in the sections of mannitol-untreated brains ($75.2\% \pm 7.9\%$) was also significantly higher than that in sections of mannitol-treated brains ($40.6\% \pm 8.8\%$) ($P < 0.05$) (Figure 4, G and S). In contrast, in the parenchyma, the number of PDGFR β /MCMV IE1-double-positive cells in the sections of mannitol-untreated brains ($34.5\% \pm 4.1\%$) was significantly lower than that in sections of mannitol-treated brains ($48.1\% \pm 5\%$) (Figure 4, H and R). The number of NG2/MCMV IE1-double-positive cells in sections of mannitol-untreated brains also significantly increased, from $36.3\% \pm 3.1\%$ to $58.4\% \pm 4.7\%$, after mannitol treatment ($P < 0.01$) (Figure 4, J and R). In MA and choroid plexus, there was a tendency toward increased colocalization of MCMV IE1 cells and pericyte markers after mannitol treatment, but this finding was not statistically significant (Figure 4S).

The comparisons of other double-stained cell numbers are as follows. In the parenchyma, the numbers of GFAP/MCMV IE1-double-positive cells were $9.6\% \pm 2.7\%$ in mannitol-treated brains and $6\% \pm 4.2\%$ in mannitol-untreated brains (Figure 4, L and R). In the parenchyma, the numbers of F4/80/MCMV IE1-double-positive cells were $3.8\% \pm 2.8\%$ in mannitol-treated brains and $0.6\% \pm 1.3\%$ in mannitol-untreated brains (Figure 4, N and R). In addition, in the parenchyma, the numbers of Olig2/MCMV IE1-double-positive cells were $1.9\% \pm 2.4\%$ in the mannitol-treated group and $2.6\% \pm 2.6\%$ in the mannitol-untreated group

Figure 3 I.V. infection of the neonatal brain with murine cytomegalovirus (MCMV). **A** and **B**: MCMV was injected into the temporal facial vein of neonatal mice. **A**: A 35-gauge needle was inserted into the facial temporal vein. **B**: Anatomical scheme of the injection site (red arrow). **C** and **D**: At 12 hours postinfection (hpi), the MCMV immediate early (IE) 1 antigen (brown) is scattered throughout the meninges and brain parenchyma (arrows). **E**: IE1-positive cells (red) colocalize with lectin-positive vascular areas (green). **F**: IE1-positive cells (red) colocalize with CD31-positive endothelial cells (green), and some IE1-positive cells localize to nonvascular areas in the meninges. **G**: Some IE1-positive cells (red) colocalize with PDGFR β -positive pericyte cells (green) (arrows). **H**: Flow cytometry analysis at 24 hpi shows that MCMV/CD31-double-positive cells account for $68.9\% \pm 16.4\%$, whereas MCMV/PDGFR β -double-positive cells account for $10.5\% \pm 2.7\%$. Four infected brains (P0) were analyzed. **I**: The MCMV genome (arrows) was detected in the vascular area and meninges at 12 hpi. **J**: MCMV IE1-positive cells (brown) scattered and increased as clusters at 72 hpi. **K** and **L**: MCMV IE1-positive cells (blue) are evident around vascular areas (CD31-positive cells; brown) of the parenchyma (**K**) and diffusely in the meninges (**L**). **M**: *In situ* hybridization shows that the MCMV genome increases and small dots are scattered around signal clusters. **N** and **O**: Double-staining for the MCMV genome (green) and MCMV IE1-positive cells (red) in the parenchyma (**N**) and meninges (**O**). **P**: At 72 hpi, MCMV IE1-positive cells (brown) are evident in SVZ. **Q**: Double-immunostaining for MCMV IE1-positive cells (brown) and nestin-positive cells (blue). **R**: F4/80-positive macrophages/microglia are prominent at the infection foci at 72 hpi. Average 25.2% of MCMV IE1-positive cells (green) double-stained with F4/80-positive cells (red). Scale bars: 840 μ m (**C** and **J**); 80 μ m (**D** and **R**); 92 μ m (**E**–**G**); 30 μ m (**I** and **Q**); 130 μ m (**K** and **L**); 16 μ m (**M**–**O**); 140 μ m (**P**).

Figure 4 Effect of murine cytomegalovirus (MCMV) infection on the mannitol-treated brain. **A:** Mannitol treatment increases Evans green staining of the brain compared with mannitol-untreated brains. **B:** MCMV immediate early (IE) 1-positive cells (black arrows) are scattered throughout the brain. **C:** The number of MCMV IE1-positive cells (337.4 ± 91.3 cells per section) in the brain at 12 hpi significantly increases compared with mannitol-untreated brains (160.2 ± 29.5 cells per section). **D:** MCMV IE1-positive cells (red) are observed in the vascular area (lectin, green) of the parenchyma. **E:** At 3 hpi, the MCMV genome (green; white arrows) is found only in the vascular area of the parenchyma (red; CD31-positive cells). **F–S:** Identification of the cell types infected with MCMV in mannitol-treated brains in the acute infection phase. **F, H, J, L, N, P,** and **Q:** The parenchyma of the brain. **G, I, K, M,** and **O:** The marginal area, including the meninges. Cells positive for MCMV IE1 (red), CD31 (green; **F** and **G**), PDGFRβ (green; **H** and **I**), NG2 (green; **J** and **K**), glial fibrillary acidic protein (green; **L** and **M**), F4/80 (green; **N** and **O**), Olig2 (**P**), and microtubule-associated protein 2 (**Q**) are shown. **R** and **S:** Comparison of the proportion of infected cells between mannitol-treated brains and mannitol-untreated brains at 12 hpi. **R:** The parenchyma of the brain. **S:** The meninges and choroid plexus. $n = 5$ (C). * $P < 0.05$, ** $P < 0.01$. Scale bars: 220 μm (**B**); 15 μm (**E**); 60 μm (**F–Q**).

(Figure 4, P and R). There were no significant differences in the numbers of GFAP/MCMV IE1, F4/80/MCMV IE1, and Olig2/MCMV IE1 double-positive cells between the mannitol-treated and -untreated groups. In the MA/choroid plexus, the numbers of F4/80/MCMV IE1—double-positive

cells were $10.8\% \pm 5.6\%$ in the mannitol-treated group and $11.9\% \pm 7.9\%$ in the mannitol-untreated group (Figure 4, O and S). Microtubule-associated protein 2—positive cells did not colocalize with MCMV IE1—positive cells in either the parenchyma or MA/choroid plexus at 12 hpi (Figure 4, Q and

Figure 5 Comparison of the extravasation of murine cytomegalovirus (MCMV) particles and fluorescent microbeads from the vascular area. **A–D:** I.V. injection of M32-enhanced green fluorescent protein (EGFP)-MCMV at 2 hours postinfection (hpi). **Arrows** indicate MCMV particles; CD31 (red), MCMV (green). The parenchyma (**A** and **B**), the meninges (**C**), and the choroid plexus (**D**). **E–H:** Fluorescent Nile red microbeads 0.1 to 0.3 μm were introduced into the neonatal mouse brain by intravascular injection. **Arrows** indicate Nile red microbeads; CD31 (green), microbeads (red). The parenchyma (**E** and **F**), the meninges (**G**), and the choroid plexus (**H**). **I:** In the parenchyma of the brain, on average, 14.0% of microbeads and 7.0% of M32-EGFP-MCMV particles were located in the extravascular area. In the meninges and choroid plexus, on average, 57.3% of microbeads and 29.8% of M32-EGFP-MCMV particles were located in the extravascular area. **J:** Heparan sulfate (red) is widely distributed in the brain. **K:** CD31-expressing cells (green) colocalize with heparan sulfate (red). * $P < 0.05$, ** $P < 0.01$. Scale bars: 22 μm (**A–D**); 75 μm (**E–H**, **K**); 900 μm (**J**).

S). These data indicate that mannitol treatment, which results in EC shrinkage and opening of TJ,³⁵ helps MCMV into passing through the endothelial barrier; however, MCMV may be trapped by the pericyte network in the parenchyma or by other cells in the MA/choroid plexus.

The Distribution of Fluorescent Microbeads Versus Murine Cytomegalovirus Particles in the Acute Infection Phase after I.V. Injection

Particle size may be the primary factor inhibiting the penetration of BBB by MCMV. We compared the permeability of the vascular area for MCMV particles, which have an affinity for receptors, with that for microbeads, which lack affinity for receptors. We injected 50 μL of M32-EGFP-MCMV (5×10^6 PFU of MCMV) and 50 μL of 0.1 to 0.3- μm fluorescent Nile red microbeads (approximately 5.75×10^9 particles) into neonatal mouse brains by intravascular injection without mannitol treatment. In frozen sections made 2 hours after injection, EGFP and Nile red were observed in and around the vascular area and MA and choroid plexus in a pattern reminiscent of MCMV IE1 expression in the acute phase of infection (Figure 5, A–H). The vascular area was stained for CD31 antigen (Figure 5, A–H). Most MCMV particles and fluorescent microbeads stayed in the vascular area, (Figure 5, A and E). In the parenchyma,

7.0% $\pm$ 1.4% of M32-EGFP-MCMV particles (Figure 5, B and I) and 14.0% $\pm$ 3% of microbeads were located in the extravascular area. In MA (Figure 5, C and G) and choroid plexus (Figure 5, D and H), on average, 29.8% $\pm$ 3.8% of M32-EGFP-MCMV particles and 57.3% $\pm$ 2.3% of microbeads were located in the extravascular area. The extravasation of M32-EGFP-MCMV particles from the vascular area was significantly decreased relative to that of microbeads in both MA ($P < 0.01$) and parenchyma ($P < 0.05$) (Figure 5I). In both M32-EGFP-MCMV particles and microbeads, the rate of extravasation out of vascular area is higher in meninges and choroid plexus than parenchyma of the brain. This indicates that the vessels of MA and choroid plexus are more permeable than those of the parenchyma. Next, we investigated whether extravasation out of vascular area in the parenchyma depends on the microbead diameter. The rate of extravasation of 1.7- to 2.2- μm microbeads is significantly less than that of 0.1- to 0.3- μm microbeads (Supplemental Figure S3, A, B, and D). On the other hand, the rate of extravasation of 0.04- to 0.06- μm fluorescent microbeads is significantly higher than that of 0.1- to 0.3- μm microbeads (Supplemental Figure S3, B–D). The majority of 0.1- to 0.3- μm fluorescent Nile red microbeads remained in the vascular lumen, suggesting that the size of the MCMV is the primary factor determining the distribution of MCMV infection throughout the brain during the acute phase of infection

Figure 6 Down-regulation of $\beta 1$ integrin decreases murine cytomegalovirus (MCMV) entry. **A:** Flow cytometry shows that a shRNA against $\beta 1$ integrin down-regulates the expression of $\beta 1$ integrin by NIH3T3 cells by 2.7-fold (red) compared with a control shRNA (blue). **B** and **C:** After MCMV infection at multiplicity of infection (MOI) of 1, MCMV immediate early (IE) 1 expression is significantly inhibited in a $\beta 1$ integrin–knockdown cell line at 5 days postinfection (p.i.) (**B**) and at 24 hours p.i. (**C**). **D:** MCMV IE1 expression (green) at MOI of 1 decreases in a $\beta 1$ integrin–knockdown cell line. **E:** MCMV genome (green) decreases in $\beta 1$ integrin–knockdown cells compared with control cells at 3 hpi. $^{**}P < 0.01$. Scale bars: 50 μ m (**D**); 20 μ m (**E**).

(Figure 5, A, E, and I and Supplemental Figure S3, B and D). However, other factors may contribute to determining the distribution of MCMV infection during the acute phase. It is known that CMV initiates infection by binding to cell-surface HSPGs.²² IHC analysis revealed that HSPGs are widely distributed in the MA and parenchyma of brain (Figure 5J). CD31-expressing cells (ECs) in parenchyma colocalized with HSPG expression (Figure 5K). This may explain why the extravasation of M32-EGFP-MCMV particles from the vascular area was significantly decreased compared with that of microbeads in both the MA and parenchyma.

Relationship between High-Level $\beta 1$ Integrin–Expressing Cells and MCMV IE1–Positive Cells in the Acute Phase of Infection

Cells such as NIH3T3 cells, which are treated with an anti- $\beta 1$ integrin–neutralizing antibody (clone DE9), exhibit

significant (>80%) reductions in MCMV infectivity, suggesting that MCMV primarily uses a $\beta 1$ integrin–specific entry pathway.²³ We performed the knockdown of $\beta 1$ integrin using an shRNA, as described in the *Materials and Methods*. Flow cytometry showed that an shRNA against $\beta 1$ integrin down-regulated the expression of $\beta 1$ integrin in NIH3T3 cells 2.7-fold compared with a control shRNA (Figure 6A). The proliferation of MCMV was significantly lower in $\beta 1$ integrin–knockdown NIH3T3 cells than that in controls at 5 days after infection (from $87.8\% \pm 1.2\%$ to $33\% \pm 6.6\%$) (Figure 6B) and 24 hpi (from $8.0\% \pm 1.5\%$ to $1.7 \pm 0.4\%$) ($P < 0.01$) (Figure 6C). Immunocytochemistry showed that the expression of MCMV IE1 was reduced in $\beta 1$ integrin–knockdown cells than in controls (Figure 6D). ISH detected reduced MCMV genome signal in $\beta 1$ integrin–knockdown cells than in control cells at 3 hpi (Figure 6E). These results confirmed the previous report that MCMV primarily uses a $\beta 1$ integrin–specific entry pathway.²³

Figure 7 Analysis of $\beta 1$ integrin expression in the neonatal mouse brain. **A**: The expression of $\beta 1$ integrin (red) is scattered throughout the presumed vascular area of the parenchyma and located in the ventricular zone and marginal area. **B–D**: CD31 (green, **B**), PDGFR β (green, **C**), and NG2 (green, **D**) colocalize with $\beta 1$ integrin (red). **E–H**: Murine cytomegalovirus (MCMV) immediate early (IE) 1 and $\beta 1$ integrin expression at 12 hours postinfection (hpi). MCMV IE1—expressing cells colocalize with $\beta 1$ integrin in the brain. **E** and **F**: Intravascular infection. **G** and **H**: Intraventricular infection. The meninges (**E** and **G**) and vascular area (**E**) expressing $\beta 1$ integrin are primary targets for MCMV infection. **F** and **H**: The choroid plexus and subventricular zone, including stem/progenitor cells, express $\beta 1$ integrin (red) that colocalizes with MCMV IE1 antigen (green). **I**: PDGFR α (red) and MCMV IE1 expression (green). **J**: Epidermal growth factor receptor (EGFR; red) and MCMV IE1 expression (green). **K**, **L**, and **M**: Flow cytometry shows that coexpression of $\beta 1$ integrin and hEF-EGFP-MCMV (GFP-positive cells) is significantly higher than coexpression of PDGFR α /MCMV or EGFR/MCMV and that the correlation between $\beta 1$ integrin and MCMV IE1 is approximately 90%, irrespective of infectious method [intraventricular infection (**K**), intravascular infection with mannitol (**L**), or intravascular infection without mannitol (**M**)]. **N**: Distribution of attached virus (M32-EGFP-MCMV) particles (green) on the surface of brain slices at 30 minutes after infection. **O** and **P**: Infection of whole brain slices (P0) with hEF-EGFP-MCMV by whole immersion. GFP-positive cells (green) colocalize with $\beta 1$ integrin—positive cells (red); colocalization shown in yellow. **P**: Magnification of double-positive cells. **Q**: Infection of whole brain slices (P21) with hEF-EGFP-MCMV by whole immersion. GFP-positive cells (green) colocalize with $\beta 1$ integrin—positive cells (red); colocalization shown in yellow. $^{**}P < 0.01$. Scale bars: 250 μm (**A**); 110 μm (**B–D**, **F**, and **H**); 60 μm (**E**, **G**, **I**, and **J**); 6.4 μm (**N**); 400 μm (**O**); 200 μm (**P** and **Q**).

Next, we analyzed $\beta 1$ integrin expression in the neonatal mouse brain. The expression of $\beta 1$ integrin is scattered throughout the presumed vascular area of the parenchyma and is also located in the ventricular zone and MA (Figure 7A). To confirm the precise location of the expression of $\beta 1$ integrin, we performed double-immunostaining of $\beta 1$ integrin and vascular markers. An endothelial marker (CD31), a pericyte marker (PDGFR β), and another pericyte maker (NG2) (Figure 7, B–D, respectively) colocalized with the high-level expression of $\beta 1$ integrin.

Next, we investigated the relationship between the expressions of MCMV IE1 and high-level $\beta 1$ integrin at 12 hpi. MCMV IE1—expressing cells colocalized with the expression of $\beta 1$ integrin in the brain after both intravascular (Figure 7, E and F) and intracerebroventricular (Figure 7, G and H) infection. After intravascular infection, the MA and vascular

area (Figure 7E) and the choroid plexus and SVZ (Figure 7F) expressed $\beta 1$ integrin colocalized with MCMV IE1 antigen. After the intraventricular infection, MA and choroid plexus (Figure 7, G and H, respectively) expressed $\beta 1$ integrin, which strongly colocalized with MCMV IE1.

Reportedly, EGFR and PDGFR α mediate the entry of HCMV strain AD169 into human fibroblasts and in some cases into endothelial and epithelial cells.^{36,37} The colocalization of PDGFR α with the expression of MCMV IE1 (Figure 7I), and the expression of EGFR with MCMV IE1 (Figure 7J) was lower than that of $\beta 1$ integrin. During flow cytometry, $59.2\% \pm 3.3\%$ of the dissociated neonatal brain cells consisted of high-level $\beta 1$ integrin—expressing cells. Flow cytometry showed that the correlation between $\beta 1$ integrin and hEF-EGFP-MCMV (GFP-positive cells) at 24 hpi was extremely high, irrespective of the infectious method

Figure 8 Inhibition of murine cytomegalovirus (MCMV) infection with functional blocking antibody against $\beta 1$ integrin *in vitro* and *in vivo*. **A:** NIH3T3 cells treated with the functional blocking anti- $\beta 1$ integrin antibody (clone Ha2/5) exhibits reductions in MCMV infectivity in a dose-dependent manner. **B:** Treatment of blocking antibody (clone Ha2/5; 10 $\mu\text{g}/\text{mL}$) to NIH3T3 cells significantly inhibits MCMV proliferation by 2.3-fold at 36 hours postinfection (hpi) in comparison with that of same isotype antibody-treated cells. **C and D:** Whole brain slices were pretreated with functional blocking anti- $\beta 1$ integrin antibody and infected with hEF-EGFP-MCMV. Infected cells in green. **Arrows** indicate representative infected cells (**C**). The number of GFP-positive cells (109.7 ± 37.3 cells per section, **D**) in the isotype-treated brain at 24 hpi is significantly higher compared to that of Ha2/5 treated brains (50.2 ± 29.2 cells per section). **E:** The number of MCMV IE1-positive cells per sections was compared between Ha2/5-treated brain and isotype-treated brain. The number of MCMV IE1-positive cells (61.2 ± 13.4 cells per section) in the brain at 24 hpi significantly decreases in comparison with that of Ha2/5-treated brains (48.0 ± 10 cells per section). $n = 5$ (**B** and **E**); $n = 9$ (**D**). * $P < 0.05$, ** $P < 0.01$. Scale bar = 240 μm (**A**); 700 μm (**C**).

[intravascular infection without mannitol ($98.2\% \pm 1.2\%$), intravascular infection with mannitol ($95.6\% \pm 2.8\%$), or intraventricular infection ($89.1\% \pm 4.7\%$) (Figure 7, K–M, respectively)]. The coexpression of $\beta 1$ integrin and MCMV (GFP-positive cells) was significantly higher than that of PDGFR α /MCMV and EGFR/MCMV (Figure 7, K–M). At 72 hpi, many MCMV IE1-positive cells existed outside the vascular area and they exhibited lower expression of $\beta 1$ integrin (Supplemental Figure S4), indicating that infection initiates from high-level $\beta 1$ integrin-expressing cells and spreads to low-level $\beta 1$ integrin-expressing cells.

To overcome the effect of the distribution of virus particles, brain slices were used to analyze the relationship between the expression of $\beta 1$ integrin and susceptibility of CMV. Brain tissue was obtained from 0-day- and 4-week-old ICR mice. Neonatal mice and adult animals were anesthetized before decapitation. The brain was cut into coronal slices of 200 μm according to Materials and Methods. Whole brain slices were immersed in M32-EGFP-MCMV and incubated for 1 hour. Figure 7N shows the ubiquitous distribution of MCMV particles attached to the surface of the brain slices. We then infected whole brain slices with hEF-EGFP-MCMV by immersion. After 24 hpi, the brain slices were immunostained with an antibody against $\beta 1$ integrin. $\beta 1$ Integrin was expressed in MA, SVZ, and vascular area, and GFP-positive cells were double-stained with $\beta 1$ integrin, particularly in the MA and vascular area of both 0- (Figure 7, O and P) and 21-day-old (Figure 7Q) brain slices. This result

emphasizes on the importance of $\beta 1$ integrin for the initial MCMV distribution of MCMV of the brain in either intraventricular infection or intravascular infection.

Functional Blocking of $\beta 1$ Integrin against MCMV Infection

NIH3T3 cells that are treated with the functional blocking anti- $\beta 1$ integrin antibody (clone Ha2/5) exhibit reductions in MCMV infectivity in a dose-dependent manner (Figure 8A). Treatment of 10 $\mu\text{g}/\text{mL}$ $\beta 1$ integrin function-blocking antibody to NIH3T3 cells significantly inhibited MCMV proliferation by 2.3-fold at 36 hpi in comparison with that of same isotype antibody-treated cells ($n = 5$; $P < 0.01$) (Figure 8B). Next, whole brain slices were pretreated with 20 $\mu\text{g}/\text{mL}$ functional blocking anti- $\beta 1$ integrin antibody or 20 $\mu\text{g}/\text{mL}$ isotype control antibody for 1 hour and infected with hEF-EGFP-MCMV. GFP-positive cells were significantly decreased in Ha2/5-pretreated brain slices in comparison with isotype control-treated brain slices ($n = 9$; $P < 0.05$) (Figure 8, C and D). Neonatal mice (P0) were injected with functional blocking anti- $\beta 1$ integrin antibody (50 μL , 20 $\mu\text{g}/\text{g}$) or isotype control antibody (50 μL , 20 $\mu\text{g}/\text{g}$) into the superficial temporal vein of neonatal mice. One hour after antibody injection, 5×10^6 PFU (50 μL) of MCMV (Smith strain) was infused into the other side of the temporal vein. The number of MCMV IE1-positive cells per section of Ha2/5-

Figure 9 Up-regulation of matrix metalloproteinase (MMP)-9 and changes in claudin-5 distribution around the area of murine cytomegalovirus (MCMV) infection. **A:** MMP activity (MMP-1 to 3, 7 to 9, and 12 to 14) was assessed using the SensoLyte 520 Generic MMP (AnaSpec Inc., Fremont, CA). At 72 hours postinfection (hpi), MMP activity in the infected brain significantly increases. **B:** Western blot analysis shows that the expression of MMP-9 up-regulates at 72 hpi. In contrast, the expression of claudin-5 does not change significantly. **C:** At 12 hpi, scant MMP-9 expression is observed around MCMV immediate early (IE) 1-positive cells (blue). **D:** At 72 hpi, MCMV infection up-regulates the expression of MMP-9 (brown) around vessels adjacent to MCMV IE1-expressing cells (blue). **E and F:** Changes in the distribution of claudin-5 after MCMV infection. **E:** Colocalization of claudin-5 (red) and MCMV IE1 (green) at 12 hpi. **F:** Changes in claudin-5 distribution (red) around MCMV IE1-expressing cells (green). * $P < 0.05$. Scale bar = 60 μm (C and D); 50 μm (E and F).

treated brain was significantly decreased compared with isotype antibody-treated brain ($n = 5$; $P < 0.05$) (Figure 8E).

Possible Mechanisms by Which CMV Infection Overcomes the Blood–Brain Barrier and Spread of Infection

Our results suggest that the majority of MCMV particles are first trapped by cells in the vascular area expressing high levels of $\beta 1$ integrin during the acute phase of intravascular infection. Although BBB works at the initial distribution of MCMV even with mannitol treatment, many MCMV IE1-positive cells were outside the vascular area, indicating the breakdown of BBB at 72 hpi (Figure 3K). The CNS excludes cells and harmful compounds circulating in the blood via BBB and blood–cerebrospinal fluid barrier, which are formed by TJ proteins around cerebral epithelial cells of the choroid plexus.³⁸ Reports on BBB disruption during congenital CMV infection are few. In a study in a mouse model of herpes simplex virus 1 encephalitis,³⁹ MMP-2 and MMP-9 activity were observed to increase,

and *in situ* zymography indicated that MMP-9 activity in the brain centered around the meninges and parenchymal blood vessels.

Furthermore, MMP-9 is up-regulated in West Nile virus infection in the periphery and brain and is partly localized to blood vessels in the brain.⁴⁰ We examined the activity of MMPs (MMP 1 to 3, 7 to 9, and 12 to 14) using the SensoLyte 520 Generic MMP assay kit. At 72 hpi, the activity of MMP in infected whole brains significantly increased ($P < 0.05$) (Figure 9A). Of the various MMPs, MMP-9 (aka gelatinase B), is thought to play an important role in BBB disruption after inflammation. After hematogenous infection, Western blot analysis revealed that the expression of MMP-9 was slightly increased (Figure 9B) and it was expressed around MCMV IE1 cells along blood vessels at 72 hpi (Figure 9, C and D). The activity of MMP induced by pathological conditions causes BBB disruption not only by basement membrane degradation but also by the cleavage of the TJ proteins occludin and claudin-5. Occludin, claudin-5, and zona occludens protein ZO-1 disruption is an indicator of the functional breakdown of the BBB.⁴¹ Claudins are integral membrane TJ proteins localized at TJs and sufficient for the formation of TJs at cell–cell contacts.⁴² Unexpectedly, the expression of claudin-5 did not change within 72 hpi of MCMV infection (Figure 9B). Although the colocalization of claudin-5 and MCMV IE1 at 12 hpi was obvious (Figure 9E), the integrity of the expression of claudin-5 around MCMV IE1-positive cells at 72 hpi was disrupted (Figure 9F).

Discussion

In animal models, intraventricular,⁴³ i.p.,^{21,44} direct placental,¹⁸ and i.v. infections into the adult tail vein^{19,33} were used to study the pathogenesis of CMV encephalitis. I.P., direct placental, and i.v. infections into the adult tail vein represent hematogenic systemic infection.

In experimental models, the earliest stages of CNS infection are not well characterized, and it is unclear whether only certain cells are initially infected. For the first time, we have analyzed the distribution of CMV particles during the acute phase of infection (termed primary viremia) after intraventricular/intravascular injection into the neonatal mouse brain. Hsu et al⁴⁵ reported that early MCMV infection administered via i.p./i.v. injection results in a distinct pattern of infection in selected cells in the mediastinal lymph nodes, spleen, and hepatocytes, but they did not discuss CNS. The appearance of infected cells in CNS within 2 days after infection has not been reported for either intraventricular or intravascular CMV infection. Furthermore, the distribution of CMV particle has not been examined. The small size of adeno-associated virus particles (20 to 25 nm in diameter) facilitates their dissemination throughout the brain in the intraventricular infection.^{46,47} It is still unclear how the relatively large CMV particles (150 to 300 nm in diameter) spread through the brain in the acute phase of infection and how the distribution of

cellular receptors and their affinity for viruses contribute to viral spread. After intraventricular infection and intravascular infection, MCMV IE1-positive cells correlated with the first distribution of microbeads (100 to 300 nm in diameter), MCMV genome signal, and MCMV particles (M32-EGFP-MCMV).

In intravascular injection of microbeads, extravasation out of the vascular area in the parenchyma depends on the microbead diameter. Microbeads that are 1.7 to 2.2 μm in diameter are less permeable than are 0.1 to 0.3 μm microbeads (Supplemental Figure S3, A, B, and D). On the other hand, 0.04- to 0.06- μm fluorescent microbeads are more permeable than are 0.1- to 0.3- μm microbeads (Supplemental Figure S3, B–D). These results, particularly those for the distribution of microbeads, support the hypothesis that CMV size (150 to 300 nm) is the primary factor restricting CMV infection.

In a comparison of the distribution of microbeads, which lack any affinity for receptors, and MCMV particles, which possess an affinity for receptors, MCMV particles were more significantly restricted to the vascular area (Figure 5), indicating that in addition to viral size, viral receptors contribute to this restriction. In addition, it showed that the vessels of MA and choroid plexus are more permeable than are those of the parenchyma (Figure 5). This difference of permeability may explain why the MA (meninges) and choroid plexus are sites susceptible to CMV infection of brain. After CMV attaches to HSPG at the cell surface,²² it engages one or more receptor(s). Higher-avidity receptors, such as CD13,⁴⁸ HLA-B27,⁴⁹ β_2 microglobulin,⁵⁰ annexin II,⁵¹ EGFR,³⁶ PDGFR α ,³⁷ or integrins⁵² have been shown to promote stable attachment, but to varying degrees. Most of these receptors have been shown to play only a minor role, or even no role,^{49,53–56} in CMV capture and entry, except for β_1 integrins and, to a lesser extent, β_3 integrins.^{23,53} In MCMV infection, the only identified entry receptor is β_1 integrin.²³ In this report, the knockdown of β_1 integrin using an shRNA significantly decreased the susceptibility to MCMV at 24 hpi and 5 days after infection. ISH revealed the reduced entry of MCMV into β_1 integrin-knockdown cells (Figure 6). IHC analysis revealed that all CD31-positive ECs, PDGFR β - and NG2-positive pericytes, ventricular zone, SVZ, meninges, and choroid plexus are the most susceptible sites in the early phase of infection (Figure 3, D–H and Q) and that all of them strongly express β_1 integrin (Figure 7, A–H). Reportedly, β_1 integrin is highly expressed in ventricular zone and SVZ, including in radial glia and NSPCs.^{57,58}

In the early phase of MCMV infection, the proportion of MCMV IE1/ β_1 integrin (high-level) double-positive cells was approximately 90%, irrespective of method of infection or mannitol treatment (Figure 7, K–M). Despite a wide distribution of MCMV particles on brain slices, the expression of MCMV colocalized with high-level β_1 integrin-expressing cells at 24 hpi (Figure 7, N–Q). In addition, the blocking studies by pretreating NIH3T3 cells, brain slices, and neonatal mice using functional blocking anti- β_1 integrin antibody before infection significantly inhibited the

infection *in vitro* and *in vivo* (Figure 8). These results confirm the hypothesis that higher expression of β_1 integrin determines the initial susceptible cells in the brain. This finding concurs with those from a previous report that stated that MCMV-positive cells (94%) at 8 and 17 hpi colocalize with β_1 integrin in both sinus and cord structures in the red pulp.⁴⁵ Although our data strongly suggested that β_1 integrin plays a major role in internalization of surface-bound CMV, there may be receptors other than β_1 integrin to be involved in CMV entry and proliferation in the brain. The possibility exists that CMV uses multiple receptors (eg, PDGFR α and EGFR) with similar cellular activity to broaden its tropism. Future work is needed to investigate this possibility.

During intravascular infection at 72 hpi, many MCMV IE1-positive cells are outside the vascular area (Figure 3K). MCMV diffuses and spreads around in adjacent cells from primary infectious foci, as shown in Figure 3, M and N. One possible mechanism for this finding is that MCMV proliferates in ECs or pericytes, causing the diffused virus to spread and causing BBB disruption. In addition, free virus may enter into the parenchyma through BBB-disrupted vessel. Another possible mechanism is that circulating infected monocytes become permissive on differentiation into tissue macrophages, enter the parenchyma through disrupted BBB foci, and then release infectious progeny within the brain parenchyma. In this experiment, approximately 1% of MCMV IE1/F4/80 double-positive cells in the parenchyma were evident at 12 hpi, which increased to approximately 25% at 72 hpi (Figure 3R). It is possible that monocytes/microglia may become newly infected at local sites or that infected monocytes may migrate through disrupted BBB from the circulation. Further investigation is required to elucidate the precise mechanism of secondary viremia.

To understand the role of the BBB in combating CMV, we enhanced BBB disruption through i.p. mannitol treatment. Previous studies have established that the mechanism of mannitol-induced BBB opening is through transient vasodilatation and the shrinkage of cerebrovascular ECs that collectively widen interendothelial TJs.^{59,60} Louboutin et al²⁹ reported that i.v. injection of recombinant simian virus 40, particularly with mannitol pretreatment, resulted in extensive expression of multiple transgenes throughout the neonatal CNS. Mannitol treatment significantly increased the number of MCMV IE1-positive cells (Figure 4C). After mannitol treatment, MCMV particles can pass through the loose TJ of ECs more easily (Figure 4, R and S). After passing through the endothelial barrier, MCMV may be trapped by the pericyte network in the parenchyma or by other cells in the MA/choroid plexus due to their high level of β_1 integrin expression. Modest differences of mannitol effect in this study suggest that the effect of the mannitol treatment is transient and may not be potent enough to open BBB for CMV.

Interestingly, β_1 integrin plays an important role in the regulation of BBB. Osada et al⁶¹ reported that EC-matrix

interactions via $\beta 1$ integrin directly affect inter-EC TJ claudin-5 expression and brain microvascular permeability. Blockade of $\beta 1$ integrin function by functional blocking antibody (Ha2/5) changes interendothelial claudin-5 expression and increases microvessel permeability. Opposite effects (CMV inhibition versus microvessel permeability increase) of functional blocking antibody (Ha2/5) may cause a significant but modest inhibitory effect of MCMV infection in the *in vivo* brain system (Figure 8E).

In this study, MCMV infection up-regulated the activity of MMPs of the whole brain (Figure 9A). The expression of MMP-9 of the whole brain was slightly increased (Figure 9B), and the expression of MMP-9 near vessels expressing MCMV IE1 was up-regulated at 72 hpi (Figure 9, C and D). Furthermore, alterations to the expression of claudin-5 in vessels was evident at 72 hpi (Figure 9, E and F).

Spindler and Hsu¹³ reviewed the viral disruption of the BBB. The host inflammatory response to viral infection can generate reactive oxygen species and reactive nitrogen species, which in turn stimulate inflammatory cytokine and MMP secretion by cells of the neurovascular unit. The activity of MMP induced in pathological conditions causes BBB disruption, both by basement membrane degradation and cleavage of the TJ proteins occludin and claudin-5. Thus viral infections of the CNS have the potential to cause BBB disruption. CMV may use a similar mechanism to disrupt the BBB. It is widely accepted that reactive oxygen species are involved in the regulation of CMV gene transcription early in infection.⁶² MCMV infection promotes both local and systemic production of proinflammatory cytokines such as tumor necrosis factor (TNF)- α and interferon- γ .⁶³ Geist et al⁶⁴ strongly suggested that CMV IE gene products up-regulate the expression of TNF- α during CMV infection. Previously, we studied a transplacental MCMV infection to establish the model of congenital CMV encephalitis and reported that the numbers of both meningeal macrophages and parenchymal microglia increased even in the uninfected areas of MCMV-infected brain due to recruitment of their precursors from other sites.⁶⁵ Expressions of nitric oxide, TNF- α , and IL-1 β , reflecting macrophage activation, increased markedly in the MCMV-infected cerebra with time after infection, but not in the mock-infected cerebra.⁶⁵

In our experimental model, F4/80-positive macrophages/microglia were prominent at the infection foci at 72 hpi (Figure 3R). These results support that there must be an inflammatory response in this present model. Alcendor et al⁶⁶ reported that CMV infection of pericytes may contribute to virus dissemination in CNS as well as neuroinflammation. Pericytes are most sensitive to TNF- α in terms of MMP-9 release and are the major source of MMP-9 at BBB.⁶⁷ MCMV infection in the brain may generate reactive oxygen species and up-regulate TNF- α from ECs, pericyte, or macrophage/microglia to induce the expression of MMP-9 around MCMV IE1-positive cells. Activated MMP-9 may induce BBB disruption to permit the extravasation of CMV particles or

infected cells around vessels. The precise mechanisms of CMV penetration of BBB may be revealed in the future.

In this study, we showed that MCMV initiates infection from high-level $\beta 1$ integrin-expressing cells in the brain after either intraventricular or intravascular infection. The initial distribution of CMV particles determines the pattern of infection in the brain. Unlike smaller adeno-associated virus particles, the relatively large size of CMV particles confers a physiological limitation on the speed at which they spread through the brain in the initial phase. High-level $\beta 1$ integrin-expressing cells lining the surface of the brain likely encounter CMV. CMV and other pathogens use $\beta 1$ integrin as an entry receptor in the brain in an ingenious manner. Integrins regulate both outside-in and inside-out signaling to maintain important cell functions related to cell adhesion, migration, and survival. Furthermore, integrins act as the global regulators of endocytosis, affecting the intracellular trafficking of growth factor receptors and endosome localization in cells. The level of the expression of $\beta 1$ integrin may determine how much CMV is internalized. The mechanisms of preventing CMV entry during the attachment phase using neutralizing antibodies or drugs may reduce the fatal congenital CNS disorders caused by CMV infection in the future.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.01.032>.

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