Systemic Immune Deficiency Necessary for Cytomegalovirus Invasion of the Mature Brain

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Cytomegalovirus (CMV) is a significant opportunistic pathogen associated with AIDS and immunosuppressive therapy. Infection of the mature central nervous system (CNS) can cause significant pathology with associated neurological deficits, mental disorders, and cognitive impairment and may have potentially fatal consequences. Using genetically immunocompromised mice, we studied mechanisms of CMV invasion into, and behavior within, the CNS. Adult immunodeficient (nude and SCID) and control mice were peripherally infected with recombinant mouse CMV expressing a green fluorescent protein reporter gene. Control mice actively eliminated acute peripheral infection and were resistant to invasion of CMV into the brain. In contrast, virus infected brains of immunodeficient mice but only after a minimum of 21 days postinoculation. After inoculation, CMV was found in circulating leukocytes (MAC-3/CD45+) and in leukocytes within the brain, suggesting these cells as a possible source of CMV entry into the CNS. CNS infection was observed in many different cell types, including neurons, glial cells, meninges, ependymal cells, and cells of cerebral vessels. Infection foci progressively expanded locally to adjacent cells, resulting in meningitis, choroiditis, encephalitis, vasculitis, and necrosis; clear indication of axonal transport of CMV was not found. Regional distribution of CMV was unique in each brain, consisting of randomly distributed, unilateral foci. Testing whether CMV gained access to brain through nonspecific vascular disruption, vascular injections of a tracer molecule revealed no obvious disruption of the blood brain barrier in mice with CMV in the brain. Results indicate the importance of host adaptive immunity (particularly T cells) in controlling entry and dissemination of CMV into the brain and are consistent with the view that virus may be carried into the brain by circulating mononuclear cells that traffic through the blood brain barrier.

Cytomegaloviruses (CMV) are important opportunistic pathogens with a very high prevalence in humans and animals (1, 31, 35). Infection is usually acquired early in life but remains latent in immunocompetent individuals. Up to 60 to 70% of the population in developed countries may be infected as early as 6 years of age (1, 21). Little is known about the cellular and molecular mechanisms that result in persistent infection, latency, and reactivation (9). However, immunosuppressed patients (e.g., those receiving immunosuppressive drug therapy and those with AIDS) can develop severe clinical disease from either a new primary CMV infection or reactivation of a latent infection (18, 21, 24, 25, 38, 40, 48, 53). CMV is a common secondary pathogen of AIDS patients, infecting more than 90% of the at-risk population. With disseminated CMV disease, virtually all organ systems can be affected, leading to mononucleosis, severe respiratory infection, liver and kidney damage, intestinal disease, and central nervous system (CNS) damage. Following the widespread introduction of highly active antiretroviral therapy in 1996, there has been a very significant reduction of CMV infection of the CNS in patients with human immunodeficiency virus (HIV) infection or AIDS. However, there are some limitations with this treatment that warrant alternative therapies (50). Highly active antiretroviral therapy may be associated with potential serious side effects; therapy has not been proven to eliminate carrier states; CMV resistance is possible; and availability, patient compliance, and cost issues may limit effectiveness (2).

In a healthy adult population CMV infection of the CNS is uncommon; however, as the population of immunosuppressed adults has continued to rise, so has the incidence of neurotropic CMV infection (21, 51, 52). CMV frequently disseminates to the CNS in late stages of HIV infection when the CD4+ T-cell count is low (19, 20). CMV is also purported to be a cofactor in AIDS dementia syndrome and can infect the same cells as HIV (5, 34, 39, 49). Clinical manifestations of neurotropic CMV infection of mature CNS may include retinitis, encephalitis, myeloradiculitis, subcortical dementia, ob- tundation, and other significant neurological deficits, with potentially fatal outcomes (1, 3, 14, 21, 33, 34, 39, 49, 51). Interaction of CMV and HIV further suppresses the immune system, thereby escalating disease. CMV encephalitis is often underdiagnosed because of the difficulty in premortem diagnosis, uncertainty about the risk factors and mechanism of dissemination, and confusion with HIV dementia (19, 20). Further, the species specificity of CMV and a scarcity of appropriate animal models to study the natural progression of peripheral CMV infection to the mature brain have limited our understanding of the relationship of systemic infection, host immunity, and CNS disease. Knowledge of CMV behavior in the CNS is essential for appreciation of site-specific neurological deficits and will provide opportunities to develop therapeuc measures against CNS infection.

Murine CMV (mCMV) has considerable gene sequence ho-
mology with human CMV as well as similar virion structure, replication cycle, systemic pathogenesis during acute infection, establishment of latency, and reactivation after immunosuppression (1, 24, 29, 31, 36). Thus, mCMV infection has been suggested as a model of human CMV infection in several contexts, including interstitial pneumonia, subclinical infection, hematological effects, chorioretinitis and encephalitis, latency, and reactivation (16, 22, 24, 35, 43; J. D. Reuter, D. L. Gomez, J. Wilson, and A. N. van den Pol, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, p. 687–688, 2001). However, peripheral inoculation with mCMV of immunocompetent or immunosuppressed adult mice has not previously been reported to lead to CNS invasion (35, 36, 41, 44).

This study tests mechanisms of peripheral CMV invasion of the CNS, defines and quantifies regional and cellular neurotropism, and reports comparative neuropathology of CMV. The use of two different strains of immunodeficient adult mice allowed us to test the hypothesis that CMV only enters the brain when the peripheral immune system, particularly the T-cell component, is depressed. Our findings that CMV infects the brain only after a prolonged period of peripheral infection, that the infection starts as random small foci of different types of cells scattered through the brain, and that it occurs only in immunodeficient and not in immunocompetent hosts give us a clearer understanding of viral invasion into the CNS and predisposing risk factors and may lead to a better understanding of a virus that is one of the leading causes of brain dysfunction in immunocompromised hosts.

MATERIALS AND METHODS

Mice. Five- to six-week-old female immunodeficient C.B17-SCID (SCID) and BALB/cAnNcr-nuBR (nu/nu) mice and immunocompetent C.B17 and BALB/cAnNCrl-BR (control) mice were obtained from Charles River Laboratories (Wilmington, Mass.) and acclimated 3 to 5 days before challenge. All mice were supplied by the supplier to be free of murine viruses, pathogenic bacteria, and endo- and ectoparasites. All mice were tested and found to be negative for anti-mCMV antibody. Control and experimental groups were housed separately in static microisolator cages on corn cob bedding, and all animal manipulations were performed in a class II A biological safety cabinet using standard microisolation techniques. Animals were housed at a temperature of 22 to 24°C with humidity of 40 to 60% and a 12-h light, 12-h dark cycle. All procedures were performed following approval by the Yale University Institutional Animal Care and Use Committee. Some of the animals were made available through the Charles River Animal Grant Program.

Assessment of SCID phenotype. Most SCID mice have very low levels of immunoglobulin. Occasionally, these mice may develop increased immunoglobulin G (IgG) levels that may affect the level of immune deficiency. To test whether immunoglobulin presence deterred CMV infection, SCID mice were bled using retro-orbital venipuncture under anesthesia prior to viral inoculation. Serum IgG levels were evaluated by a sandwich enzyme-linked immunosorbent assay (ELISA) for isotype detection. Ninety-six-well Immunosorb microtiter plates (Nunc/Nalgene, Naperville, Ill.) were coated with goat anti-mouse IgG (whole molecule) antibody (Cappel/ICN, Costa Mesa, Calif.) at a concentration of 1 µg/ml. The plates were incubated overnight at 4°C, washed, and blocked with 3% gelatin (Sigma, St. Louis, Mo.). IgG standard (purified mouse myeloma protein IgG; Cappel/ICN) was prepared at a concentration of 1.1 µg/ml. Serial twofold dilutions were plated in duplicate for a standard curve from 0.55 to 106 µg/ml. Standard and sera were incubated at 37°C for 1 h. Plates were washed and then incubated for 1 h at 37°C. Following washing, the plates were developed for 5 min at room temperature with 3,3′,5,5′-tetramethylbenzidine substrate (KPL, Gaithersburg, Md.). Stop solution (KPL) was added, and the optical density at 450 nm was read (MRK ELISA Reader; ThermoLabSystems, Chantilly, Va.). The standard curve was plotted. Any levels in serum greater than or equal to 0.4 µg/ml were considered positive.

Virus and infections. Recombinant and wild-type mCMV (strain K181 MC.55 [ie2+/GFP−]) strongly and rapidly expresses green fluorescent protein (GFP) under control of the human elongation factor-1α promoter inserted at the immediate-early gene (IE2) site (45). Expression of GFP enables the direct detection of mCMV in living and fixed, unstained tissue. Recombinant (mCMV-GFP) and K181 wild-type mCMV (mCMV-wt) stocks were passaged in 3T3 cells (American Type Culture Collection, Manassas, Va.). Cells were harvested, semipurified, titrated, using plaque assays, and stored in aliquots at −70°C.

In initial experiments, mice were anesthetized with subcutaneous ketamine (100 mg/kg) and xylazine (10 mg/kg) and inoculated with 100 µl of mCMV-GFP (4.4 × 106 PFU) injected into the lateral tail vein. Additional studies were conducted assessing the effect of the route of infection on subsequent virus dissemination. For these studies, mice received 20 µl of intranasal (1.0 × 106 PFU), 150 µl of intraperitoneal (1.0 × 106 PFU), or 100 µl of intravascular (4.4 × 106 PFU) mCMV-GFP inoculants. Mice were assessed daily for clinical illness, and any moribund mouse was humanely euthanized. At various experimental endpoints (3, 10, 14, 21, 28, 32, or 35 days postinoculation [dpi]), mice were euthanized with carbon dioxide gas and immediately perfused transcardially with borax-buffered saline followed by 4% paraformaldehyde in phosphate buffer (PFA).

For control purposes, an additional experiment was conducted using the K181 mCMV-wt (4.4 × 106 PFU or 4.4 × 104 PFU) injected intravascularly into SCID and control mice. Mice were sacrificed and perfused at 21 dpi.

To assess whether mature CNS cells of normal immunocompetent mice were inherently resistant to infection or whether the lack of infection was due to rapid viral clearance by active systemic immunity, SCID and control mice received 1.5 µl of mCMV-GFP (2.0 × 106 to 4.4 × 106 PFU) injected intracranially in the left cerebrum 1 to 2 mm off midline, 3 to 4 mm caudal to the orbit to the 25-gauge burr hole using a Hamilton syringe. Mice were euthanized 2, 4, and 6 days later with carbon dioxide gas and immediately perfused transcardially with heparinized saline followed by 4% PFA.

Quantification of virus. We examined tissues using plaque assay, direct visualization for GFP, and immunostaining. In brief, mice were euthanized and immediately transcardially perfused with sterile, cold PBS to remove potential virus contained within the blood. Tissues were collected and snap-frozen in liquid nitrogen. A 10% (wt/vol) homogenate was made in Dulbecco’s modified Eagle medium, and diluted aliquots were incubated on 3T3 cells for 1 h at 37°C on a rocker. The suspension was removed, and cells were overlaid with 0.95% SeaKem agar in 2× growth medium (45% Dulbecco’s modified Eagle medium, 45% L15, fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin; Life Technologies, GibcoBRL, Grand Island, N.Y.). After 5 to 6 days of incubation at 37°C with 5% CO2, plaque assay monolayers were fixed with PFA, stained with crystal violet, and visualized for cytopathic effects.

GFP Detection. For stereotropically perfused brain, lung, liver, and spleen were immersed overnight in 4% PFA and cryoprotected in 10% and then 30% sucrose in cryoprotectant and stored in the dark at −20°C. GFP fluorescence was recorded using fluorescein isothiocyanate filters on a Zeiss Axioscope, captured with a Zeiss AxiosCam digital camera. Contrast and intensity of images were corrected using Zeiss AxioVision digital imaging software and Adobe Photoshop.

Validation of reporter protein. Polyclonal antisera were generated by inoculating BALB/c mice with 106 PFU of mCMV-GFP through intraperitoneal and intranasal routes, and this was repeated after 4 weeks. Sera were collected and pooled 2 weeks following the second inoculation. The specificity and sensitivity of the polyclonal antibody were assessed against both recombinant and K181 mCMV-wt viruses using several methods. 3T3 mouse fibroblasts were grown to 75% confluency either in 25-cm2 culture flasks or on four-well plastic chamber slides (Nunc/Nalgene) and infected with mCMV-GFP or mCMV-wt at a multiplicity of infection of 5 to 10. Following 1 h of adsorption at 37°C on a rocker, the infected monolayers were washed with phosphate buffer and replaced with growth medium. Forty-eight hours after infection, cells from culture flasks were harvested and fixed with 4% PFA for antigen dot blot analysis.
or immunofluorescence titration (IFA). Viral antigen extract was obtained by washing isolated infected cells, followed by repeated freeze-thawing to rupture cells. Total protein was quantified using Bio-Rad protein assay, and antigen load was standardized, titrated, applied to nitrocellulose paper, allowed to dry, and blocked in 5% defatted milk. Dot blots were performed by spotting 1 µl of a 1:200 dilution of the following: rat anti-mouse CD45R/B220, and mouse anti-IL-2R (Jackson ImmunoResearch Laboratories, Inc.) and developed with VECTASTAIN ABC kit and DAB (Vector Laboratories Inc., Burlingame, Calif.). IFA endpoint titration was done on infected cells transferred to 12-well spot slides (Erie Scient. Inc., Portman, N.H.). Cells were incubated with titrated primary in sera, washed, and incubated with a 1:500 dilution of goat anti-mouse immunoglobulin conjugated to Cy3 (Chemicon International, Temecula, Calif.). To localize immunoreactive viral proteins, mCMV infection in individual chamber slides was stopped after 6, 21, 30, and 48 h of infection. Cells were fixed with 4% PFA and stained with a 1:200 dilution of antisera, followed by a secondary antibody (above); fluorescence was detected with a rhodamine filter set (590 nm).

Immunohistochemistry (IHC) of GFP-positive brain and select tissue sections using the generated anti-CMV sera was done to analyze the specificity and sensitivity of the GFP reporter protein in mCMV-GFP-infected tissue. Controls included omission of primary or secondary antisera, the use of tissue from immunocompetent mice where no GFP or immunostaining for viral proteins was expected, and the use of noninfected tissue.

Tissue staining. Select tissues were fixed in 10% buffered formalin for histopathology. Brains from a cohort of SCID and control mice were examined for histopathologic markers of infection using hematoxylin and cosin. CNS pathology for remaining mice was analyzed by direct observation of unstained sections during GFP analysis. We identified infected cell types using several methods. Morphological determination of infected cells was easily done by visualizing brain cells as GFP highlighted characteristic cellular processes. Identification of infected cells within the CNS was confirmed using commercial antibodies against cellular antigens. Briefly, tissue sections were incubated overnight at 4°C with monoclonal mouse anti-Neu antibody (1:500), rabbit anti-gal fibrillary astrocyte protein (1:200), rat anti-myelin basic protein (1:200) (Chemicon International) or monoclonal rat anti-mouse CD31 (1:200) (PECAM-1; BD Biosciences, Pharmingen, San Diego, Calif.). Tissues were washed three times in phosphate buffer plus 0.3% Triton-X. Secondary antibodies, including goat anti-rabbit, anti-rat, or anti-mouse IgG conjugated to cosin, Cy3 (1:500) (Chemicon International), or AlexaFluor 350 goat anti-mouse, anti-rat, anti-rabbit IgG (1:100) (Molecular Probes, Eugene, Ore.), were applied for 1 h at room temperature. All tissues were initially blocked with 5% normal goat serum–1% bovine serum albumin–0.3% Triton-X for 1 h before incubation with primary antibody. Anti-bodies were diluted in 1% normal goat serum–1% bovine serum albumin–0.3% Triton-X.

Viremia measurement. To detect the presence of virus in the blood (viremia), DNA was extracted from pooled sera and isolated buffy coats of infected or uninfected animals using a QIAmp DNA Blood Mini Kit (Qiagen, Valencia, Calif.) and quantified for semiquantitative PCR using previously described PCR primers (6). To identify cell-associated CMV in peripheral blood, whole blood was collected using heparinized capillary tubes and samples were pooled by infection group. Erythrocytes were lysed by resuspension in 1.6% ammonium chloride, 0.2% potassium bicarbonate, and 0.03% EDTA tetra sodium with a final adjusted pH of 7.2. Isolated leukocytes were washed three times in phosphate buffer, and cytological preparations were made of condensed cells. Cytological analysis was done under fluorescent light to identify GFP-positive leukocytes. Identification of positive cells was done by immunohistochemical staining with a 1-h incubation of a 1:200 dilution of the following: rat anti-mouse CD45RB/B220, rat anti-mouse MAC-3 (BD Biosciences Pharmingen), and mouse anti-IL-2Rβ or mouse anti-CD3 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). Slides were then washed and incubated in a 1:500 dilution of secondary anti-rat or anti-mouse Cy3-conjugated antibody (Chemicon International).

Assessment of BBB integrity. Since a large proportion of mice exhibited CMV meningitis, a subgroup of 21-day mCMV-GFP-infected SCID mice were given two injections of 30 µg of peroxidease through the lateral tail vein 1 h apart (Worthington Biochemical Corp., Lakewood, N.J.) to detect whether virus was disseminating to the brain through nonspecific breakdown of the blood brain barrier (BBB) (11). Three hours after the initial peroxidase injection, mice were euthanized and perfused (as described above). GFP-positive cells were quantified using ImageJ software (version 1.25; National Institutes of Health) to analyze and contrast, standardize background threshold, and calculate mean fluorescence density from CMV-mediated GFP expression. Previous tissue culture experiments had shown a significant positive correlation between viral load and GFP fluorescence. Because the distribution of infectious foci was asymmetrical in the brain and did not resemble the generalized, diffuse pattern seen in other systemic tissues, mean GFP fluorescence per brain tissue section was highly variable. For meaningful comparison of organ viral density in Fig. 1, mean GFP fluorescence density in the brain was computed by determining a mean fluorescence density standard curve based on the number of infected cells per tissue section in peripheral organs. The average number of infected cells per section was then fit to the standard curve.

**RESULTS**

Validation of reporter protein and antisera. Sensitivity and specificity of GFP as a reporter for mCMV infection were analyzed in infected control and infected organ tissue. Infected cells began expressing GFP in both nuclear and cytosolic compartments as early as 6 h after infection of mouse fibroblasts (Fig. 1A to D). We tested the relationship between inoculation titer and the number of cells showing GFP expression 24 h later. The number of cells that expressed GFP was highly correlated (r² = 0.99; P < 0.0001) to the titer of the inoculation (Fig. 1H). Antisera identified viral antigens in a selective and sensitive pattern. Labeling was primarily seen for cytosolic viral proteins from early and both cytosolic and nuclear from late stages of replication. Dot blot and IFA analysis confirmed the specificity of the antisera for both mCMV-GFP and mCMV-WT viral proteins. No immunostaining was detected in noninfected mouse cells (Fig. 1G). Similarly, only mouse brain infected with CMV was immunopositive; noninfected control mouse brain showed no immunoreactivity (not shown). IFA titration of the antisera was effective through a dilution of at least 1:12,500.

GFP expression was validated as a marker for mCMV-infected organs using IHC and plaque assay. A strong correlation (r = 0.95; P < 0.0001) was observed between brain cells expressing the GFP viral reporter and those positively staining for viral proteins with polyclonal anti-mCMV sera (Fig. 1E and F). Occasionally, positive immunohistochemical staining of cells was associated with small or no GFP infection foci. This may represent early-stage infection with inadequate production of GFP needed for detection by fluorescent microscopy. In tissue culture, detectable GFP fluorescence of infected cells preceded changes in cellular morphology caused by cytopathic viral effects. In general, detection of GFP expression was sensitive and provided a very rapid means of identifying mCMV-infected cells.

Peripheral mCMV inoculation. mCMV replication and progressive dissemination to brain was studied using the reporter protein as an indicator of virus-infected cells in multiple experimental paradigms consisting of more than 200 mice (see Tables 1 and 2 for partial summary). No differences in clinical progression, pathology, or organ distribution were seen between intravascular wild-type (4.4 × 10⁴ PFU) and recombi-
FIG. 1. GFP expression as a valid marker for CMV-infected cells. (A to D) mCMV-GFP infection (multiplicity of infection = 10) in 3T3 mouse fibroblasts after 6 h. (A) GFP expression; (B) IHC staining; (C) DAPI (4',6'-diamidino-2-phenylindole) staining; (D) phase contrast. Arrows highlight examples of co-staining cells infected with virus. (E) Section of brain (5 μm thick) showing GFP expression in mCMV-infected brain cells. (F) Same section as in panel E, showing immunostaining of infected cells with mouse polyclonal anti-mCMV antibody. Arrowheads show cells...
TABLE 1. Disseminated mCMV infection as a result of immune deficiency

<table>
<thead>
<tr>
<th>Mouse phenotype</th>
<th>dpi</th>
<th>CNS infection</th>
<th>Active systemic infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Severity</td>
<td>Incidence</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0/2</td>
<td>0</td>
</tr>
<tr>
<td>Nude</td>
<td>21</td>
<td>4/10</td>
<td>1–3</td>
</tr>
<tr>
<td>SCID</td>
<td>3</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0/5</td>
<td>0</td>
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<tr>
<td></td>
<td>21</td>
<td>10/15</td>
<td>1–4</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>9/10</td>
<td>3–4</td>
</tr>
</tbody>
</table>

* Intravenous challenge with 100 µl of virus (4.4 × 10⁵ PFU) in the lateral tail vein.
* Number of infected mice/total no. of mice.
* Severity scores: 0, negative; 1, 1 to 2 infected cells per focus (CPF); 2, 2 to 20 CPF; 3, 21 to 50 CPF; 4, >50 CPF. Negative mice from 21 and 35 dpi are not scored.

TABLE 2. Direct intracranial mCMV infection in SCID and immunocompetent control mice

<table>
<thead>
<tr>
<th>Virus dose* (PFU)</th>
<th>dpi</th>
<th>SCID mice</th>
<th>Control mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Severity</td>
<td>Incidence</td>
</tr>
<tr>
<td>2 × 10⁴</td>
<td>6</td>
<td>5/5</td>
<td>2–3</td>
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<tr>
<td>4.4 × 10³</td>
<td>2</td>
<td>3/3</td>
<td>1–2</td>
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<tr>
<td></td>
<td>6</td>
<td>5/5</td>
<td>4</td>
</tr>
</tbody>
</table>

* Intracranial challenge with 1.5 µl of virus.
* Number of infected mice/total no. of mice.
* Severity scores: 1, low number of infected cells; 2–3, intermediate number of infected cells; 4, high number of infected cells.

1). Infection patterns resembled that of a hematogenously spread pathogen, occurring in a multifocal, diffuse pattern centered in areas of increased vascularity, although viremia levels at this stage of infection were below the level of detection. Inoculated control mice consistently cleared active mCMV-GFP infection within 21 days of inoculation, evidenced by the lack of fluorescence in any tissue (Fig. 2A to F). The systemic organ virus density in SCID mice temporally increased throughout the entire length of the study with an increase in the number and size of infection foci as indicated by a strong increase in the number of infected cells and overall fluorescence. Levels of organ GFP fluorescence were positively correlated with the number of GFP-expressing infected cells in the organ (Fig. 2H). Systemic organ infection was greatest 35 days following inoculation, with the greatest number of infected cells seen in the spleen followed by lung and liver.

**CNS disease.** CNS infection occurred both in nude and SCID mice and was initially detected after a prolonged interval of approximately 21 days following mCMV-GFP infection. The severity of CNS infection was most consistent in SCID mice. In tests of SCID mice, circulating virus was detected in MAC-3/CD45⁺ IL-2R/B220/CD3⁺ mononuclear cells beginning at 14 dpi using immunocytochemistry to identify cell type and GFP expression as a marker for CMV infection (Fig. 3A to C). Isolated leukocytes were pooled by group to allow detection of these rare infected cells. In those groups with the highest level of CMV in the brain, we found circulating infected cells, while in those groups without CMV in the brain, we were unable to detect any infected cells. CMV-infected MAC-3-positive mononuclear cells were also associated with infection foci in the brain (Fig. 3D to G). During the acute infection stage (0 to 14 days), we were unable to detect virus from sera or isolated peripheral blood mononuclear cells using virus isolation in cell culture via 50% tissue culture infectious dose; however, a low level of viremia was detected in later stages of infection using semiquantitative PCR of pooled sera and peripheral blood mononuclear cell samples (data not shown).

Mice developed overt abnormal behavioral signs (photopho-
and ataxia) beginning 28 days following infection. Although 100% of mice infected with mCMV-GFP developed systemic 
infected in peripheral organs are similar in both control and SCID phenotypes in acute infection but significantly increase over time in SCID mice (G) while disappearing completely in control mice (I) by 21 days following inoculation. Group sample sizes are indicated (N). Dissemination to the CNS occurred only in SCID mice, beginning 21 days dpi and increasing through 35 dpi. (H) Counts of fluorescent cell number per unit area in the lung after mCMV-GFP infection. A strong positive correlation was found between organ green fluorescence and the number of infected cells. The x axis depicts days following peripheral infection. (G and I) Error bars, SEM. Scale bar = 100 μm.

FIG. 2. Temporal quantification of mCMV infection in tissue following peripheral inoculation. As more cells became infected with the GFP-expressing CMV, organ fluorescence increased. Significant multifocal, diffuse fluorescence is present in multiple systemic organs in SCID mice but not control mice 21 days following inoculation. Shown are photomicrographs of GFP-labeled cells in spleen (A and D), lung (B and E), and liver (C and F). (A to C) Control mouse; (D to F) SCID mouse. The mean numbers of infected cells in peripheral organs are similar in both control and SCID phenotypes in acute infection but significantly increase over time in SCID mice (G) while disappearing completely in control mice (I) by 21 days following inoculation. Group sample sizes are indicated (N). Dissemination to the CNS occurred only in SCID mice, beginning 21 days dpi and increasing through 35 dpi. (H) Counts of fluorescent cell number per unit area in the lung after mCMV-GFP infection. A strong positive correlation was found between organ green fluorescence and the number of infected cells. The x axis depicts days following peripheral infection. (G and I) Error bars, SEM. Scale bar = 100 μm.

bria and ataxia) beginning 28 days following infection. Although 100% of mice infected with mCMV-GFP developed systemic infection, the degree of CNS infection of nude and SCID mice varied, with an occasional mouse showing no detectable CNS infection (Table 1). This variability was not a result of immunoglobulin depression, as nude (T-cell-deficient) and SCID (T- and B-cell-deficient) mice alike developed CNS disease. To determine if SCID mice showing no CNS infection had greater levels of humoral immunity, immunoglobulins were measured. On average, 20% of 5- to 6-week-old female CB-17 SCID mice were determined to have positive immunoglobulin levels, and the remaining 80% had strongly depressed immunoglobulin levels. None of the detected antibody was mCMV-antigen specific. No correlation was found between brain levels of CMV and levels of circulating immunoglobulin levels, as many of the SCID mice with detectable immunoglobulin developed CMV encephalitis.

To test the hypothesis that a high level of peripheral infection might promote CNS infection, we compared peripheral infection levels with those of the CNS. Relative numbers of cells infected in systemic organs were strongly correlated to the number of cells in the CNS ($r = 0.92; P < 0.0001$). Distinct infection foci within the CNS occurred in a nonsymmetrical multifocal pattern, unlike that seen systemically, where infection occurred diffusely in many scattered cells throughout other organs (Fig. 4G). CNS foci locally expanded to neighboring cells over time, and growing foci could contain both neurons (characterized by two or three primary dendrites and an axon) and glial cells (characterized by large numbers of short processes arising from the cell body). Dissemination within the CNS was greatest when viral infection included the ventricular system or meninges. Similar results were found with GFP-expressing recombinant CMV and with wild-type CMV, confirming that the recombinant CMV behaved similarly to wild-type CMV in the context of invasion of the CNS.

Infectious foci were seen equally throughout the forebrain, midbrain, and hindbrain and increased proportionately for the duration of infection (Fig. 5). Infectivity of virus was confirmed by plaque assay. CMV foci and GFP-expressing infected cells were assessed throughout the entire brain. Twenty-one days after challenge, $13.8 ± 4.4$ (mean ± standard error of the mean [SEM]) CMV foci occurred per mouse brain versus $25.0 ± 8.5$ CMV foci per mouse brain 35 days after inoculation. The number of cells per CNS infection focus was significantly increased in later periods of incubation: $42.7 ± 19.7$ infected cells per focus at 21 days versus $137.4 ± 52.4$ infected cells per focus at 35 dpi (Fig. 5A and B). Total numbers of infected cells
brain cells were significantly greater in SCID mice after 35 days than in earlier periods (421.2 ± 214.5 total infected cells per brain at 21 days following inoculation versus 3,769.6 ± 1,549.2 total infected cells per brain at 35 days following inoculation [Fig. 5C]).

GFP expression was detected in many different cell types of the brain. Many infected cell types were identified using fluorescent microscopy to view characteristic morphology highlighted by GFP fluorescence. Additional cell types were identified using the specific location within the brain and immunohistochemical staining. Neuronal cell bodies, dendrites, and axons showed GFP expression indicative of viral infection of neurons (Fig. 6). Although neuronal axons did express significant GFP fluorescence, no evidence of axonal release of CMV was seen. Other infected cell types included tanycytes, astrocytes, meninges, and smooth muscle cells in vessel walls. Giant

FIG. 3. MAC-3-positive infected mononuclear cells in mCMV-infected SCID mice. (A) Cytological preparation of GFP-expressing monocytes found in blood 14 days following peripheral mCMV inoculation. Selective red MAC-3 immunostaining (B) and composite (C) showing dual staining of infected cells. The long arrow highlights mCMV-infected, MAC-3-positive double staining; the short arrow illustrates non-mCMV-infected, MAC-3-positive monocytes; and arrowheads indicate non-mCMV-infected, MAC-3-negative cells. (D) Brain section (5 μm thick) with GFP-expressing monocytes in the olfactory bulb of SCID mice 21 days following peripheral mCMV inoculation (unstained). (E) Selective MAC-3 staining; (F) DAPI staining; (G) composite of panels D to F. (H) Low-power magnification composite of mCMV infection foci and monocytes. Note focal aggregation of inflammatory cells (red) corresponding to infection foci (green); arrow shows same cell as presented in panels D to G. Bar = 25 μm (A to C), 10 μm (D to G), 50 μm (H).
cells, caused by CMV-mediated fusion of cellular membranes, were found in areas of strong infection. Although each brain showed different regions of infection, within the SCID mouse group, most regions in the brain, including the olfactory bulb, striatum, cortex, hippocampus, thalamus, hypothalamus, cerebellum, dorsal peduncles, choroid plexus, anterior and posterior commissures, superior colliculus, and medulla, showed some degree of infection (Fig. 4A to F). Spinal cord was not analyzed. Pathological changes included meningitis, choroiditis, encephalitis, giant cell formation, necrosis, and neuronal cell death (Fig. 7).

Toward understanding viral entry into brain, we tested the hypothesis that mCMV might infect olfactory bulbs through anterograde movement of virus from olfactory epithelium in immunocompromised mice. Direct intranasal application of virus in SCID mice resulted in olfactory epithelium infection and pneumonia, but no direct CNS infection was detected in olfactory nerve or its targets in the olfactory bulb through 35 days of incubation. Direct movement of the virus through the olfactory nerves to brain was not seen by direct examination for GFP. Both peritoneal and vascular inoculation resulted in widespread viral dissemination in peripheral organs and both routes of infection lead to asymmetrical localized CNS disease.

**BBB.** Since vascular dissemination may be critical for viral entry into the CNS, we tested whether entry was a result of nonspecific breakdown of the BBB integrity. HRP injected into the vascular system is a sensitive indicator of BBB integrity. Staining of brain parenchyma was negative for HRP in SCID mice following virus inoculation, even with concomitant viral

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**FIG. 4.** Mouse CMV infects different brain regions. mCMV infection of the CNS in SCID mice 21 to 35 days following peripheral inoculation, detected with GFP reporter. (A) Olfactory bulb (granular cell and external plexiform layers). (B) Dorsal peduncle. (C) Focal striatal infection, asymmetrical and localized. Multiple cell types are infected with mCMV, including astrocytes, neurons, and glial cells. (D) Extension of GFP from neuronal cell body to dendritic processes. (E) Infected cells of the choroid plexus (arrows). (F) Localized region of infection near the central canal above the medulla; infected tanyocyte processes (arrow). Unstained sections. Bar = 25 μm (C), 50 μm (B and D), or 100 μm (A, E, and F). (G) Schematic diagrams of coronal sections through immunodeficient mouse brain illustrating CMV-infected foci following peripheral inoculation. CMV-infected cells (dots) are asymmetrical in location and extend by local expansion rather than following neuronal tracts. Brains typically have several independent and unrelated sites of infection, suggesting that a unique event is occurring at each location, allowing virus to penetrate the CNS and replicate locally.
GFP expression in the same sections (Fig. 8). As a positive control demonstrating successful detection of HRP leakage in the brain, we studied the circumventricular organs (Fig. 8G and H). These have previously been reported to show HRP labeling after escape from the vascular system in normal mice due to fenestrated blood vessels (11). We found HRP in the circumventricular organs, but found no CMV in the same area. Active uptake of peroxidase was seen in several peripheral tissues, indicating successful circulation of the label.

To determine if CMV infections might result in occlusion of capillary blood flow, we perfused mice with heparinized saline followed by paraformaldehyde, with the working assumption that blood cells should be washed from the brain if no vascular compromise was present. Some regions of the brain where a large CMV-infected site was situated also showed substantial accumulations of erythrocytes (Fig. 8A to C). No vascular congestion was detected when only a small number of CMV-infected cells were found or in non-CMV-infected brains.

**Intracranial inoculation.** Since virus did not disseminate to the CNS of immunocompetent adult control mice after peripheral inoculation, we asked whether mature CNS cells of these normal immunocompetent mice were inherently resistant to infection, or whether the lack of infection was due to rapid viral clearance by active systemic immunity. To address this question, both SCID and immunocompetent age-matched control mice were inoculated with direct intracranial injection of mCMV-GFP under anesthesia. No adverse effects were seen postprocedure, and both SCID and immunocompetent control mice continued to behave normally during the acute stage of cerebral infection (6 days). Control mice, although immunocompetent, were equally susceptible to acute cerebral infection following CNS injection (Table 2). Susceptible cell types on the CNS were similar to those seen in the disseminated SCID mouse model described earlier in Results. Infection occurred in a number of distinct cell types, including astrocytes, oligodendrocytes, meninges, neurons, ependymal cells in the walls of the ventricles, and choroid plexus. Often, virus-induced multinucleated giant cells were seen, signifying cell membrane fusion. Infection was localized to cells surrounding the injection site and did not disseminate to distant areas of the brain unless the injection site was associated with the lateral ventricle or meningeal infection occurred. No significant differences between GFP-expressing cell bodies per tissue section in immunodeficient and control mice were observed. Within the 6-day study period, we found no evidence for CMV dissemination out of the CNS following intracranial inoculation. Thus, CMV can infect many distinct cells of the CNS in both normal and immunodeficient brains, and neither active systemic immunity nor innate resistance of mature neurons prevented acute localized cerebral infection when mCMV was delivered directly into the brain.

**DISCUSSION**

In these studies, mCMV invaded the brains of SCID and nude adult mice, but no CNS infection was found in immunocompetent adult mice. These results support the hypothesis that a depressed system immune system is necessary for CMV invasion of the mature brain. We believe this is the first report in adult mice of experimental CMV dissemination and active...
infection from the periphery into the CNS. Our data strongly support the hypothesis that CMV enters the brain at a number of independent sites due to multiple events. Typical infection in the CNS included a number of randomly located, unilateral foci of infected cells, and occurred only after substantial numbers of infected cells were present in peripheral organs, potentially acting as seeding sites for dispersal into the brain. Data showing the lack of CMV infection at any time point under conditions of the experimental methods used in the normal adult brain indicate the critical importance of systemic host immunity in controlling CNS entry and dissemination of mCMV after parenteral inoculation.

**CMV enters the CNS.** Although previous studies have examined CMV in normal and immunodeficient mice, active dissemination into the brain has not been reported (35, 36, 41, 44). This lack of detection may have been due to short intervals of inoculation, immune status of the animals, directed focus on non-CNS organs, or simply missing small foci of infection. Latent CMV DNA has been reported from homogenized mouse CNS tissue (15); however, neither cellular tropism nor pathology was identified, and there is a possibility that endothelial cells, vascular walls, or nucleated cells within the blood, not brain cells, were the source of latency (23, 28). In our study, some brains showed no signs of infection, even in brains of
peripherally infected immunodeficient mice. One strong advantage of the GFP viral reporter used in the present study is it allowed us to readily examine large numbers of serial sections without the necessity of immunohistochemical procedures. This aided us in detecting small CMV foci, including one-cell foci. Using the immunodeficient mouse, we have made some potentially interesting observations, examined below.

We did not find overwhelming viremia in the circulatory system, nor obvious breakdown of the vascular system, nor transport of the virus via the olfactory nerve, nor primary viral infection of the circumventricular organs through the local weak BBB, suggesting some other mechanism of viral entry into the brain, perhaps via infected circulating leukocytes. Infected endothelial cells and monocytes have been suggested as vectors for viral dissemination (23, 30). However, nucleated cells are thought to make one pass through the vascular system, and the half-life in circulation is approximately 1 min (30). Thus, it would be unlikely that any CMV-infected cells would remain very long in the blood, making detection of viremia in early stages difficult. Viral DNA has been recovered from circulating monocytes related to bone marrow infection (35). In addition, we have identified active infection occurring in circulating cells. Viral infection of leukocytes corresponds with high virus titers in internal organs such as the lung, liver, and spleen (12), similar to the high organ viral densities we report in the present study. In our study, disseminated, asymmetric foci of CMV infection were identified throughout the brain, with no apparent interrelationship to one another. This pattern suggests multiple independent seedings of the CNS from a circulatory source; if the virus spreads within the brain, one would expect some relationship between the foci, and this was not found except after ventricular involvement. These sites of infection may result from transmigration of the observed infected circulating leukocytes across the BBB and release of

FIG. 7. Pathology of the brain in SCID mice following peripheral inoculation of mCMV. (A) Medial entorhinal cortical encephalitis with loss of normal architecture and liquefactive necrosis (arrow). (B) Posterior commissure perivascular infiltration and intramural inflammation (arrows). (C) Severe meningitis indicated by cellular infiltrate and loss of normal architecture (arrow). (D) Olfactory bulb necrosis; arrowheads indicate karyopyknosis and karyorrhexis. Hematoxylin and cosin staining. Bar = 20 µm (D), 50 µm (B and C), or 100 µm (A).
FIG. 8. Integrity of the BBB following mCMV infection. (A) Unstained cryosection exhibiting GFP expression from mCMV infection (arrows).
(B) DAB-labeled erythrocytes (arrows) illustrating congestion in infected brain region, but no leakage into CNS parenchyma. (C) DAPI staining of section showing cellular nuclei. (D) Unstained cryosection showing GFP. (E) Negative peroxidase staining of infected parenchyma. (F) DAPI staining. (G) Positive control for peroxidase leakage from the vasculature in the circumventricular organ (arrow), with no labeling in surrounding tissue. (H) Phase-contrast image of the same area shown in panel G. Panels A, B, and C show the same section; panels D, E, and F show the same section. GCC, genu of the corpus callosum. Bar = 100 μm (A to F) or 50 μm (G and H).
virus. Our finding of mCMV-infected monocytes in the brain helps support this association, although we cannot rule out the possibility that these cells may have become infected within the CNS. Alternately, virus could enter the brain from infected endothelial cells or from local temporary disruptions of the BBB. The circumventricular organs are regions of the CNS with weak BBBs that allow the escape of large molecules such as HRP from the vascular system (11); it is noteworthy that we found no obvious signs of CMV infection in the circumventricular organs, including the median eminence, subfornical organ, or organum vasculosum of the lamina terminalis, except when the ventricular system as a whole showed signs of CMV infection.

Previous studies with other viruses have shown that in general, viremia load and duration correlate with CNS invasion (42). Low-titer primary viremia typically spreads infection to other sites before a higher-titer secondary viremia develops (42). Human CMV may disseminate to the CNS by hematogenous routes (52). However, detection of CMV viremia in early stages of infection is generally very difficult and suggests consistently low levels. Although peripheral organs showed rapid signs of infection, there was a considerable time lag after peripheral inoculation before infection was detected in the CNS. During this period, levels of CMV infection in peripheral organs of immunodeficient mice continually escalated. We found no indication of direct CNS infection near the time of peripheral inoculation, suggesting that when the CNS infection did occur at a later time point, it was due to release of later generations of replicating virus. The extended interval between virus inoculation and CNS infection supports the aforementioned concept that systemic viremia, possibly fed by systemic virus replication, leads to infection of CNS. Humans infected with human immunodeficiency virus frequently have detectable CMV viremia in late stages of infection, and infection of endothelial cells has been reported (19, 20, 37). Mouse CMV also infects endothelial cells in the brain (32), indicating that CMV may infect CNS vessels prior to parenchymal CNS infection. We found infected vessels within the brain and local extension into parenchymal tissue. Furthermore, brain tissue culture studies showed infection of endothelial cells indicating that neuronal vascular tissue is permissive to infection (44). These data suggest that even during immunodeficiency, the BBB and other intrinsic mechanisms kept the brain free of virus infection for a considerable period. Possible intrinsic mechanisms that serve to protect the brain from CMV infection may include variation in cellular constituents, virus receptor expression, innate cellular immunity, or the ability to support CMV replication in brain compared to peripheral organs.

**Multiple sites of CMV infection.** Our data support the view that CMV enters the brain at a number of independent sites due to single events. The pattern of CMV is similar to that reported for CMV in immunosuppressed humans. With human neurotropic CMV infection, diffuse seeding of cellular nodules, with a higher incidence in gray matter (basal ganglia, diencephalon, and brain stem), and foci of cytomegalic cells accompanied by necrotizing ventriculo-enhepalitis and polyradiculomyelitis are found (9). Similar pathology is observed with the neonatal guinea pig model, including perivascular infiltrates, vascular endothelial swelling, subependymal infiltrates, and sporadic focal leptomeningitis (7, 8, 17). This suggests that even after some virus may have entered the brain, therapeutically reducing general viremia may reduce further infiltration of CMV to other brain regions, an important consideration for clinical treatment.

Typical infection in the CNS included a number of randomly located, asymmetric foci of infected cells. These occurred in all regions of the brain rostrally from the olfactory bulb and caudally to the medulla and cerebellum, included most cell types in the brain, and appeared to start from independent events followed by local diffusion of the virus to neighboring cells. A single focus of infection might include both nearby neurons and glia. Foci were consistently unilateral unless they were directly on the midline and were suggestive of movement of virus from one cell to another by local diffusion from virus released from cell bodies or proximal processes, as suggested by studies of viral movement in brain slices (46). Unlike alphaherpesviruses such as herpes simplex that may be transported intraxonally and subsequently released (54), we found no obvious indication of axonal transport and release of this betaherpesvirus from one region to another. General dissemination of CMV was found only in cases where virus penetrated the ventricular system and, in those cases, spread throughout the lateral and medial ventricular system.

**Immune defense against CMV in brain.** Our model provides unique opportunities to study the relationships between invading viruses, systemic host defenses, and immuno-privileged sites. The availability of selective, immunocompromised mouse genotypes dramatically enhances the opportunities for testing and manipulation, not readily available with other models. CNS disease in adult nonhuman primates has been described in the form of case reports (4), but the use of primates for these studies is difficult and most virus laboratories do not have facilities for primates. Coinfection with simian immunodeficiency virus is necessary to induce immune deficiency, but this virus is pathogenic and often leads to encephalitis and giant cell formation. Additionally, primates are at risk of multiple, overlapping opportunistic diseases. Together, these factors may complicate interpretation of CMV-related damage. A recent report that uses rhesus CMV offers an intriguing model to study CMV in the developing brain; however, adaptation of this to study viral invasion of the mature CNS would be faced with limitations similar to those outlined above (13). Other murine and guinea pig models of CMV infection using direct intracranial injection are potentially useful to study virus behavior within the brain (7, 8, 27, 35, 45). However, direct injection accelerates CNS dissemination by circumventing natural host defenses including the BBB, loads the tissue with a highly concentrated focus of virus, and may create iatrogenic parenchyma lesions with associated disruption of the BBB. Thus, these intracranial inoculation models may not be ideal for prospective study of immune regulation and methods of dissemination to the mature CNS.

In addition to the immunodeficient mature brain studied here, CMV is also a problem virus in the developing brain, but viral entry mechanisms may be different. Parenteral (intraperitoneal) inoculation of developing neonatal mice and guinea pigs causes widespread CMV dissemination, including to the CNS (17, 24, 27). However, the mechanism of virus entry into
the developing brain is in part due to an undeveloped immune system coupled with an ineffective BBB, which is permissive to viral invasion until approximately 10 days of age (27).

In contrast, the BBB in adults is effective at keeping virus out of the brain, as shown by the long incubation period prior to the first signs of CNS infection in the present study; together these results suggest that virus may enter the adult brain by a mechanism different from that by which it enters the developing brain. In addition, developing neurons, both in vivo and in vitro, are inherently more susceptible to CMV infection than are mature neurons, independent of the status of the systemic immune system (46, 47).

The lack of CMV infection in the normal adult brain under the experimental conditions used here indicates the critical importance of systemic host immunity in controlling CNS entry and dissemination of mCMV after parenteral inoculation. SCID mice are defective in T and B cells, and nude mice are defective in T cells of the adaptive immune system, while both have normal levels of natural killer cells (10; Handbook on genetically standardized Jax mice, 5th ed., Jackson Laboratory, Bar Harbor, Maine). Innate immunity was insufficient to block CMV dissemination, as both SCID and nude phenotypes showed CMV in brain, even when individual mice had increased levels of immunoglobulin. Without adequate cellular immunity, systemic virus density progressed and disseminated to immunologic privileged sites. The various levels of prevalence of CNS infection in these mice resemble variations of neurotropic human CMV infection in immunosuppressed patients. As many as 90% of AIDS patients are thought to be systemically infected with CMV, and 50% may have CNS disease caused by opportunistic CMV (26, 51, 52). In parallel, in our experiments with immunodeficient mice, a small number showed no CMV infection in the brain. This difference may reflect variations in cellular immunity, or may be related to intrinsic cellular factors, as disease progression is seen in patients with strong humoral immunity. Individual variations in gene expression or physiologic performance may influence susceptibility to virus infection directly, by changing cellular milieu, or indirectly, by altering blood pressure, capillary permeability, or innate immunity.

Overview. Using adult immunocompromised mice, we tested several hypotheses relating to natural modes of viral entry and behavior in the adult brain. We found multiple independent infection focus sites possibly due to CMV-infected circulating monocytes. A critical observation of the present study was that whereas immunodeficient mice showed considerable levels of delayed CNS infection, CMV infection was consistently and dramatically absent from the brains of immunocompetent mice. Our data show striking parallels with CMV infection of the brains of immunosuppressed humans. The significant delay in neurotropic infection supports the view that early treatment of systemic CMV infection should reduce the probability of the virus getting into the brain. Moreover, the study of CMV in the immunodeficient mature mouse will be useful in future work to test the hypothesis that restoration of adaptive immunity will enable the immune system to attenuate or prevent and possibly clear the CNS of CMV infection.

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