Brain Trauma Enhances Transient Cytomegalovirus Invasion of the Brain Only in Mice That Are Immunodeficient[▽]

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Cytomegalovirus (CMV) is one of the most common viral pathogens leading to neurological dysfunction in individuals with depressed immune systems. How CMV enters the brain remains an open question. The hypothesis that brain injury may enhance the entrance of CMV into the brain was tested. Insertion of a sterile needle into the brain caused a dramatic increase in mouse CMV in the brains of immunodeficient SCID mice inoculated peripherally within an hour of injury and examined 1 week later; peripheral inoculation 48 h after injury and a 1-week survival resulted in only a modest infection at the site of injury. In contrast, uninjured SCID mice, as well as injured immunocompetent control mice, showed little sign of viral infection at the same time intervals. Direct inoculation of the brain resulted in widespread dispersal and enhanced replication of mCMV in SCID brains tested 1 week later but not in parallel control brains. Differential viremia was unlikely to account for the greater viral load in the SCID brain, since increased mCMV in the blood of SCID compared to controls was not detected until a longer interval. These data suggest that brain injury enhances CMV invasion of the brain, but only when the adaptive immune system is compromised, and that the brain's ability to resist viral infection recovers rapidly after injury.

Cytomegalovirus (CMV) is a common virus found in the majority of adult humans. In contrast to the normal mature brain, where CMV infections are uncommon (15, 16), in individuals compromised by human immunodeficiency virus (2, 22, 30) or during medical immunosuppression associated with organ transplants (10, 21), CMV infections are common. CMV acts as an opportunistic virus that is associated with substantial complications once it has entered the central nervous system (CNS) (1, 10, 11, 15, 30, 32, 33, 34). A number of mechanisms keep viruses such as CMV out of the brain; however, once inside the brain, viruses can spread rapidly, particularly within the ventricular systems. Outside the brain, T- and antibody-generating B lymphocytes of the adaptive immune system, as well as other cells of the systemic immune system, including natural killer and macrophage/monocytes, fight and reduce CMV infections (1, 4, 8, 23). The factors that influence CMV entrance into the adult brain have not been clearly elucidated. In the fetal human brain, in the absence of a developed blood-brain barrier (BBB), CMV can enter the brain and cause substantial neurological disease (1, 3, 9).

Mouse and human CMV each have genomes of 235 kb of double-stranded DNA, have similar tissue affinity, similar viral morphology and biology, similar ability to achieve long-term latency, and colinear gene sequences with different nucleotide sequences within many genes (15, 16, 25), suggesting that mouse CMV (mCMV) in many respects parallels its human counterpart.

A number of papers studying CMV dispersal in normal and immunocompromised mice reported CMV infection in a num-

ber of peripheral organs but did not report CMV in the brain (16, 20, 23). When a CMV with a green fluorescent protein (GFP) reporter was used for peripheral infection, CMV-infected neurons and glia were found in the brain, but only in mice with a deficient systemic immune system and only after periods exceeding 3 weeks postinoculation (19). Even at 3 weeks postinoculation, some SCID mice showed no infections in the CNS (19), indicating that even the brains of immunodeficient mice can resist CMV infection for a considerable period. This raises the question as to what factors might contribute to CMV infections of the brain.

In the present study, the hypothesis that CNS trauma enhances the ability of peripheral mCMV to enter and spread in the mature brain is addressed. Adult normal and immunodeficient mice were compared after peripheral inoculation with mCMV. To facilitate histological localization of cells that were infected with CMV, a recombinant mCMV that expressed GFP in infected cells was used, as previously described (26, 27). This recombinant virus behaved similarly to wild-type virus (19, 26, 27).

MATERIALS AND METHODS

Mice. In these experiments, immunodeficient SCID mice and normal immunocompetent mice were used. In some mice, to produce a modest brain injury, mice were deeply anesthetized with ketamine and xylazine (100 and 10 mg/kg [body weight]); a thin sterile 30 gauge hypodermic needle was lowered 3 mm into the brain and then immediately withdrawn. No fluid and no virus was injected into these mice. At intervals of less than an hour to several days after the brain injury, virus was administered by tail vein injection of no more than 100 μ l.

In another group of mice, after anesthesia with ketamine and xylazine, mCMV (2×10^4 PFU) was injected directly into the brain in a volume of 1 μ l. At 1 week after the intracranial inoculation, the mouse was given an anesthetic overdose, and the brains were harvested for histological or quantitative PCR (qPCR) analysis (see below).

To study viremia, mCMV (10^6 PFU in 100 μ l) was injected into the tail vein, and the blood was sampled retro-orbitally in anesthetized mice at intervals of up to 3 weeks postinoculation. Use of mice in these experiments was approved by the Yale University Committee on Animal Use.

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Virus. An mCMV was used that expressed a GFP reporter gene in infected cells. The GFP was inserted at the ie2 site. The virus appears to behave similarly to wild-type virus and has been used and described previously (19, 26, 27). Vesicular stomatitis virus (VSV) that expressed a GFP reporter gene in the first gene position (18) was also used as a control virus for one experiment.

qPCR. For comparisons of viral load, qPCR was used. DNA was purified by using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. To quantify mCMV load, a probe that measured the mCMV IE-1 DNA sequence was detected by qPCR carried out with a TaqMan gene expression assay (ABI part no. 4331348; forward primer, CTCCTCTACCTGTCTCTGTCTTTCA; reverse primer, ACGCTCTCACTGGAATATAAGAGT; FAM dye probe, CACCCACACAGAACAC). The results were normalized relative to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase; FAM/MGB probe, non-primer limited; RefSeq NM_008084.2; probe exon location, 3; amplicon size, 107 bp [ABI catalog no. 4352932E]). A standard curve for known concentrations of virus was generated. Further details of the qPCR are given elsewhere (28).

Histology. Mice were given an overdose of pentobarbital (Nembutal) and perfused transcardially with physiological saline, followed by 4% paraformaldehyde. Brains were harvested and cut in 10- to 20- μ m-thick coronal sections on a cryostat; all comparisons were made from sections of the same thickness. Sections were picked up on gelatin-coated glass slides. Sections were examined in an Olympus IX70 fluorescence microscope. Photomicrographs were made with a Spot digital camera (Diagnostic Imaging). Images were corrected in Adobe Photoshop for contrast and brightness.

RESULTS

CMV enters the brain at sites of injury in immunodeficient mice. In the first set of experiments, a sterile 30-gauge hypodermic needle was inserted into the brains of SCID mice to generate a thin puncture wound; nothing was injected into the brain. The wound was small, and no effect on behavior, motor activity, or food intake was found in the succeeding days. Mice were inoculated via intravenous tail vein injection with mCMV ($100\ \mu\text{l}$ of 10^6 PFU) within 1 h of brain trauma; the data for one mouse were not included because virus injection into the tail vein worked poorly. At 6 to 8 days postinoculation, mice were deeply anesthetized and perfused transcardially with saline, followed by 4% paraformaldehyde. Sections (14 μm thick) were cut through the brain on a cryostat. All seven SCID mice inoculated in the tail vein showed strong signs of CMV infection in the brain, detected primarily by GFP expression (Fig. 1A) and confirmed in some cases with immunostaining with antisera against mCMV described elsewhere (19). The number of infected green cells at the needle track was large in comparison to the rest of the brain. On many sections, hundreds of infected cells could be found at the needle track, with no cells more than 200 μm in distance from the track (Fig. 1A). In some mice (three of seven), the needle track occurred near the lateral ventricle. If signs of infection were found in the adjoining ventricle (Fig. 1B), then the virus spread to other ventricular regions such as the third ventricle (Fig. 1C), underlining the role of the ventricular system in virus spread. Ventricular infection resulted in further dissemination of the virus into brain cells adjacent to the ventricular ependyma.

Infected cells included glial cells and neurons, as confirmed with immunostaining with glial fibrillary acidic protein and neuron-specific enolase or NeuN, respectively. The location of infected cells was dependent on the placement of the needle injury; infected neurons and glia were found in the cerebral cortex, hippocampus, striatum, thalamus, and hypothalamus (Fig. 1D and 2A).

In three mice, two puncture wounds were made on opposite

sides of the brain. One mouse showed infected cells in both puncture wounds. However, in serial sections of the other two mice, one puncture wound in each mouse showed hundreds of infected cells per section, whereas serial section analysis of the wounds on the contralateral side of the same brain revealed no infected cells. This lack of infection in some needle tracks suggests that, even after injury, virus penetration into the brain may be a rare event.

CMV does not enter the injured immunocompetent brain. To determine whether the entrance of CMV into the CNS was dependent on a deficient immune system, normal immunocompetent mice were examined with the same experimental protocol. Eight days after inoculation with mCMV, five of five normal mice showed no GFP in the brain, despite analysis of serial sections cut through the site of the needle insertion. No sign of mCMV infection or GFP expression was found even in the scar of the needle (Fig. 2C). The lack of strong mCMV infection in immunocompetent mice is consistent with previous findings from this and other labs (19). As a further control, five SCID mice were inoculated with mCMV, but no needle was inserted into the brain. None of these mice showed any sign of green fluorescence or mCMV in the brain when examined histologically one week later.

These control data are consistent with a previous report, where we found no mCMV in the SCID brain within a week of peripheral inoculation but did find that mCMV did disseminate to the brain only after a minimum of 21 days postinoculation and at a time when peripheral organs showed high levels of mCMV infection (19).

To determine the relative viral load in the brain, the experiments above were repeated, with a needle injury followed within an hour by intravenous inoculation of mCMV. Brains were harvested a week later, and the whole brain was homogenized and used for qPCR analysis of the relative levels of viral infection. All SCID mice ($n = 5$) showed mCMV in the brain; five of seven control mice showed no detectable mCMV in the brain, and two controls showed low levels of mCMV. SCID mice ($n = 5$) as a group had a mean of 108.2 ± 74 mCMV genomes/million brain cells, which is 20-fold greater than the mCMV infection in the whole brain of normal control mice ($n = 7$), which had 5.5 ± 3.9 mCMV genomes/million cells). In this analysis, the entire brain was used, including blood within the brain blood vessels, and this blood could potentially contribute to the small amount of virus found in some of the normal brains. The question of mCMV in the blood is addressed further below.

Small number of infected cells in SCID within 2 days of inoculation. The findings presented above raise the question of whether the difference in CMV infection between normal and SCID mouse brain is that the virus does not enter the brain in normal mice or that it ultimately faces an environment hostile to virus spread. To address this question, we made a needle injury as described above and, within 1 h, injected the virus in the tail vein of normal and SCID mice. After 2 days, the time it would take the virus to enter the brain and express the GFP reporter gene, mice were given an overdose of pentobarbital, and the brains were harvested. In normal mice ($n = 4$) 116 brain sections were studied; two cells showed expression of the GFP reporter gene, but most

FIG. 1. (A) Typical example of a needle track in the cortex of a SCID mouse made 1 h after intravenous inoculation with mCMV and killed 1 week later. Many GFP-labeled infected cells can be seen along the site of injury. Scale bar, 50 μm . (B and C) If mCMV entered the lateral ventricle in a SCID mouse brain as shown here, it spread to the ependyma throughout the ventricular system (B), including the third ventricle (C). Scale bars, 40 μm . (D) Typical neurons infected with mCMV in the cortex. Two cells with pyramidal shapes are indicated by arrows. Scale bar, 18 μm .

sections showed no labeled cells (Fig. 3B). All SCID mice tested ($n = 4$) showed GFP-positive cells, although only a few were found in each brain. In 109 histological sections, a total of 11 GFP-positive cells were found, about one infected cell per 10 sections (Fig. 3A), a nonsignificant difference. This experiment shows that mice show signs of infection in the brain at early times of 2 days after inoculation and that the number of infected cells is small.

Recovery after injury reduces CMV in brain. In the experiments described above, mCMV was administered peripherally within an hour after brain injury. To test the hypothesis that the brain injury heals relatively quickly and blocks CMV entry, the same amount of mCMV (100 μl containing 10^6 PFU) was given at 48 h after the same needle penetration injury used above; SCID mice were again given 6 to 8 days for the virus to infect the brain, and then were given an overdose of anesthetic.

FIG. 2. (A) In SCID mice inoculated intravenously 48 h after trauma and killed a week later, groups of infected GFP-positive mCMV-infected cells (green arrow) can be found selectively along the needle track (red arrow). Scale bar, 50 μ m. (B) On the opposite side of the brain (right), no virus could be detected. (C) When a similar needle injury was made in a normal immunocompetent control mouse, no GFP fluorescence indicating CMV presence was detected a week after intravenous mCMV injection. This image shows the needle track (arrow), where the red is due to the autofluorescence of traumatized cells. Scale bar, 45 μ m. (D) In a number of SCID mice, despite a needle injury (red, arrows), no CMV infection was found. Scale bar, 45 μ m.

In this group, only three of eight mice showed viral infection at the site of injury (Fig. 2A) but not on the uninjured contralateral side of the brain (Fig. 2B); the remaining five mice showed no viral infection at the needle track (Fig. 2D). All eight mice showed virus infection in the peripheral organs.

These findings suggest that the damage to the BBB heals sufficiently within 48 h to reduce viral penetration at the site of injury. To test this hypothesis, the integrity of the BBB was

tested at the two time points by injecting the dye Evans blue (0.5 mg in physiological saline) into the tail vein at 1 or 48 h after the same injury used in the virus experiments. Evans blue and related tracers only enter the brain at sites of breakdown in the BBB and in the circumventricular organs such as the median eminence (5). Uninjured brains showed no dye labeling in the cortex. In contrast, in four penetrations of a 30-gauge needle through the cortex the number of dye-labeled cortical

FIG. 3. Mice received a needle wound in the brain, followed within an hour by mCMV inoculation. Brains were harvested 2 days later. These micrographs show the occasional mCMV-infected cell in SCID mice (A), and the apparent absence of many infected cells in normal control mouse brains (B). Scale bar, 30 μ m.

cells decreased by 90% between 1 and 48 h, a statistically significant decrease ($P < 0.01$; Student t test) (Fig. 4). These data are consistent with the view that the injury-induced breakdown in the BBB shows substantial but not full recovery by 2 days after injury.

FIG. 4. (A) Evans blue-labeled cells when the dye was injected intravenously 1 h after injury. Scale bar, 20 μ m. (B) Fewer cells are seen when the dye was injected 2 days postinjury. (C) Bar graph showing the number (mean $\pm$ the SEM) of dye-labeled cells at 1 and 48 h postinjury.

To examine further mCMV entrance into the brain, SCID mice were inoculated peripherally first (100 μ l containing 10^6 PFU mCMV) and 2 weeks later received the same puncture wound in the brain that is described above. Whereas in the initial experiment described above, all SCID mice showed mCMV in the brain, in this set of experiments mCMV infection was found in the minority of cases. In two of seven mice, mCMV was detected in the area of the puncture wound. In the remaining five mice, no sign of mCMV infection was found near the needle tracks; all seven mice showed mCMV outside the brain.

mCMV in blood over time. The finding of more virus in SCID than in normal brains could be due to a greater level of viremia peripherally in SCID mice, leading to increased exposure of the brain. To test this, we collected blood retro-orbitally at 3-, 7-, 14-, and 21-day intervals after inoculation from 20 mice. The blood was analyzed for the relative level of mCMV in the blood by qPCR to quantify mCMV genomes per unit blood. The graph shown in Fig. 5 shows the change in the virus load in the blood over a period of 3 weeks. The two groups of mice showed very similar levels of virus in the blood at 3 and 7 days postinoculation; these two points are important since they overlap the period during which a substantial rise in viral load was found in the injured SCID brain. By day 14, normal control mice ($n = 12$) showed a decrease in viremia ($P < 0.05$; Student t test), whereas SCID mice ($n = 8$) showed little change. At 21 days postinoculation, SCID mice showed an increase in viremia, and controls showed a further decrease; the level of mCMV in the blood was substantially different in the two groups at this last time point ($P < 0.05$; Student t test).

mCMV increase after intracranial inoculation in SCID mice. In the experiments described above, virus was administered by peripheral tail vein inoculation. Little if any virus was found in normal brain compared to strong infection in SCID mice. To determine whether virus would spread more aggressively in the brain in SCID mice, control ($n = 9$) and SCID ($n = 6$) mice received direct intracranial injections of mCMV. Control mice consisted of five Swiss-Webster and four BALB/c mice; since the two strains of control mice showed no differences, their data were pooled. A week later, the mice

FIG. 5. Viremia. The mCMV load in blood was tested over time for 3 weeks by using qPCR to quantify mCMV genomes. SCID means plus the SEM are indicated by black bars, and normal control mice are indicated by gray bars. By day 7 postinoculation, little difference was seen between SCID and controls. By day 21, viremia had increased in SCID but decreased in control mice. The data are expressed as mCMV genomes/ml.

were given an overdose of anesthetic and compared using histology and qPCR analysis. Two of the SCID mice died before the end of the week from brain infection, and the brains were not included in further analysis but probably included high titers of virus; none of the controls died or showed signs of neurological dysfunction. Both SCID and normal mice showed infected cells within the brain, but the number of infected cells was dramatically different. For the most part, in normal brains a small number of infected cells was found around the site of injection into the brain. In contrast, SCID brains showed virus-infected cells in many brain regions, indicating a spread from the initial inoculation site. The difference between control and SCID mice could be visualized by examining the whole intact brain under fluorescent illumination; the control mice showed little or no infected cells, whereas the SCID mice showed many bright green GFP-expressing cells in the cortex, hypothalamus, and olfactory bulb (Fig. 6).

The difference between SCID and control mice was confirmed independently with qPCR. Similar to the experiment in the paragraph above, mCMV (2×10^4 PFU) was injected directly into the brain, and the brains were harvested a week later. SCID mice ($n = 4$) showed $179,329 \pm 38,554$ (standard error of the mean [SEM]) mCMV genomes/million brain cells, a level that was 102.5-fold greater than for control mice ($n = 9$) with normal immunity that had only $1,750 \pm 1,126$ mCMV genomes/million cells. These qPCR and histological data together suggest that, independent of peripheral differences, once in the brain, mCMV replicates and disperses to a much greater degree in SCID brains than in control brains.

As a final proof-of-principle experiment, two SCID mice received an intracranial needle penetration, followed within an hour by intravenous inoculation of an unrelated virus, the negative-strand RNA virus, VSV ($100 \mu\text{l}$ of 5×10^7 PFU). Since VSV replicates faster than mCMV, after 4 days the animals, instead of waiting the standard 7 days postinoculation were given an overdose of anesthetic, perfused with fixative, and histological sections were examined. Similar to the finding

FIG. 6. mCMV was injected directly into the brains of SCID and control mice. One week later the external surfaces of the brains were examined with an upright fluorescent stereomicroscope. (A and B) SCID mice showed a large number of GFP positive infected cells in the cortex. (C and D) Control brains showed few infected cells. Scale bar, $60 \mu\text{m}$.

with mCMV, VSV was detected selectively at the site of needle injury in one animal and appeared to be starting to spread away from the injury (Fig. 7). Interestingly, virus was detected only in one part of the needle track, with a substantial percentage of the needle track showing no virus, corroborating the view that virus entry into the brain, even after injury, may be a rare event. Histological sections of the second mouse showed the needle injury in the brain, but no VSV was detected. We previously showed, in the context of oncolytic targeting of brain tumors, that following 10 days of recovery after brain damage, VSV does not enter the brain (17). These data are consistent with the view that viruses can take advantage of a breakdown in the BBB but that the probability of virus getting into the brain is still a rare event.

DISCUSSION

The experiments undertaken here use a simple model of brain injury, a puncture wound. Since this would cause a breakdown in the BBB, CMV or CMV-infected cells, perhaps monocytes or macrophages (12, 14, 19), could then enter the brain.

FIG. 7. VSV enters the brain after injury. At 4 days after needle injury to the brain, followed by intravenous inoculation with VSV, brains were examined. This photomicrograph shows the needle injury in orange (orange arrows) and, in the lower part of the needle track, some cells (green arrows) infected with VSV. Note that a good deal of the needle track shows no infected cells. Scale bar, 45 μ m.

Here, the loss of integrity of the BBB, coupled with a depressed adaptive immune system, are the critical factors allowing CMV infection of the CNS. Little infection was found in normal mice that received a brain injury, indicating that the injury itself was not sufficient to produce substantial brain infection. Similarly, SCID mice with no brain injury showed little infection of the brain in the 1-week test period used. A possibility that cannot be discounted is that injured cells, or reactive microglia or astroglia, in the brain may have enhanced susceptibility to mCMV, although this is unlikely to account for the strong dispersal of mCMV to uninjured areas of the SCID brain over time. At 3 weeks postinoculation, but not earlier, some nontraumatized immunodeficient mice were previously found to show mCMV in the brain, but many mice with a depressed adaptive immune system still showed no virus in the brain even at this time point where most other organs were infected (19); this underlines the potential role for injury in enhancing the probability of virus infections in the brain. The BBB showed a substantial 90% recovery between 1 and 48 h postinjury. This correlates strongly with the decrease in mCMV-infected cells when inoculations were done at 2 days after injury, providing further support for the view that breakdown of the BBB is a critical event in allowing mCMV to enter the brain. mCMV-infected brains were found in seven of seven SCID mice with inoculation at 1 h postinjury but only in three of eight SCIDs with inoculations at 48 h postinjury.

Several lines of evidence suggest that although penetration of the virus into the brain may be a rare event, once inside the

brain, the virus spreads vigorously. First, in two of three mice receiving two puncture wounds each, one wound in each mouse showed hundreds of nearby infected cells, whereas a similar wound on the contralateral side of the brain showed no infected cells. Second, in SCID mice with brain trauma and peripheral inoculation that were tested 48 h after inoculation, only one of 10 sections showed signs of a single infected cell, with the other sections showing no signs of infection; this supports the perspective that the probability of mCMV getting into the brain may be low. Third, direct injection of the virus into the brain led to a manyfold greater spread and increase of virus from the injection site in SCID than in control mice. Fourth, analysis of viremia in the blood showed little difference in SCID compared to controls on days 3 and 7 postinoculation; in contrast, when the virus was injected directly into the CNS, brain levels of virus were >100-fold greater in SCID than controls over the same 1-week period. This last point suggests that peripheral increases in mCMV are unlikely to be the primary cause of the strong mCMV infection in the brain of SCID mice 1 week after inoculation.

The data reported here may generalize to other types of brain injury, including stroke, edema, skull trauma, or head injury that can also cause a temporary loss of the integrity of the BBB and may therefore allow CMV penetration into the brain. Although CMV infections of the brain in immunocompromised individuals are common, a number of immunocompromised humans show no sign of CMV in the brain for many years (2, 21, 22, 29, 30). The study here suggests that one factor that may increase the probability of CMV infection in the immunocompromised brain is the occurrence of some form of brain injury, possibly only a minor injury. Asymptomatic microbleeds have been described in the "normal" human brain (7, 24). Other opportunistic microorganisms, including fungus, bacteria, or other viruses that increase in the face of a depressed immune response might also increase the probability of minor breakdowns in the BBB (31), and human immunodeficiency virus (HIV)-AIDS is associated with a dysfunctional BBB (6). Other CNS problems such as multiple sclerosis, ischemia, and stroke can cause temporary increases in the permeability of the BBB (13). All of these factors that disrupt the BBB may enhance the probability of CMV entry and infection of the brain. Although CMV is the focus of the present report, an unrelated virus, VSV, also entered the brain after a needle injury, suggesting that the findings here may also generalize to other viruses that normally would not cross the BBB but, similar to CMV, could cross after injury.

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