**REVIEW ARTICLE**

# Brain slice culture for analysis of developmental brain disorders with special reference to congenital cytomegalovirus infection

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**ABSTRACT** **Cytomegalovirus (CMV) is the most significant infectious cause of congenital abnormalities of the central nervous system (CNS) with variation from the fatal cytomegalic inclusion disease to functional brain disorder. The phenotype and degree of the brain disorder depends on infection time during the developing stage, virulence, route of infection and the viral susceptibility of the cells. The pathogenesis of the CMV infection to the CNS seems to be strongly related to neural migration, neural death, cellular compositions and the immune system of the brain. To understand the complex mechanism of this disorder, we used organotypic brain slice cultures. In the brain slice culture system, migration of CMV-infected neuronal cells was observed, which reflects infectious dynamics *in vivo*. Neural progenitor cells or glial immature cells in the subventricular zone and marginal area are most susceptible to murine cytomegalovirus (MCMV) infection in this system. The susceptibility declined as the number of immature glial cells decreased with age. The immature glial cells proliferated in brain slice cultures during prolonged incubation, and the susceptibility to MCMV infection also increased in association with the proliferation of these cells. The brain slice from an immunocompromised mouse (Beige-SCID mouse) unexpectedly showed lower susceptibility than that of an immunocompetent mouse during any prolonged incubation. These results suggest that the number of immature glial cells might determine the susceptibility of CMV infection to the brain, independent of the immune system. We reviewed recent findings of CMV infection to the brain from the perspective of brain slice cultures and the possibility that this system could be a useful method to investigate mechanisms of congenital anomaly of the brain.**

**Key Words:** brain slice culture, congenital cytomegalovirus infection, developmental brain disorder

## INTRODUCTION

Cytomegalovirus (CMV) is the most significant infectious cause of congenital anomalies of the central nervous system (CNS). It is caused by an intrauterine infection in humans (Weller, 1971; Ho, 1991), with an average incidence of 1% of all live births (Stagno *et al.*, 1986; Demmler, 1991). It is estimated that approximately 5-10% of infected infants have generalized cytomegalic inclusion disease at birth, with symptoms such as microcephaly, perivascular calcification, and microphthalmia (Becroft, 1981; Bale, 1984; Cinque *et al.*, 1997). Another 10% of infected infants have subclinical congenital infection, and will subsequently suffer from brain disorders, including mental retardation, sensorineural hearing loss, visual disorders, seizures and epilepsy (Pass *et al*, 1980; Conboy *et al*, 1986).

Because studies of human subjects have obvious limitations, experiments with animal models for congenital CMV infection have been reported using guinea pig CMV (Kumar and Nankervis, 1978), murine CMV (Tsutsui, 1995) and simian CMV (London *et al.*, 1986). We have developed model systems for brain abnormalities induced by infection of mouse embryos with murine CMV (Tsutsui and Naruse, 1987; Tsutsui *et al.*, 1993; Li and Tsutsui, 2000). To further investigate the pathogenesis of CMV infection in the CNS, we chose the brain slice system. Brain slice cultures provide a useful experimental system because they preserve the three-dimensional architecture and local environment of neurons, glia, microglia, oligodendroglia to a greater extent than dissociated cell cultures while still preserving the ability to perform experimental manipulations and observations (Lo *et al.*, 1994). Organotypic slice cultures of the brain have been used increasingly to study

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the neuronal death, mechanism of cell migration, and function of microglia, myelination, electrophysiological activities, synapse plasticity and virus infectivity. Our group has applied this unique system for the analysis of cytomegalovirus to the brain for the first time (Shinmura *et al.*, 1999). Here we will review the current scientific findings of congenital cytomegalovirus infection to the brain from the perspective of the organotypic brain slice culture.

## PREPARATION OF THE BRAIN SLICE

The whole brain, dissected from a mouse and kept in cold Hanks' balanced salt solution (HBSS; Gibco BRL, Grand Island, N.Y.) on ice (**Fig. 1A**), was fixed on a stage with instant glue and cut in a coronal direction at 400-μm thickness with a microslicer (Dohan EM, Kyoto, Japan) (**Fig. 1B**). Slices were transferred to porous transparent membranes (Millicell-CM, 0.4 mm; Millipore, Bedford, Mass), which were floated on a culture medium in a six-well plate (Becton Dickinson, Franklin Lakes, N.J.) as reported previously (Shinmura *et al.*, 1999) (**Fig. 1C**). The medium consisted of 50% minimum essential medium (MEM; Gibco BRL), 25% HBSS, 25% heat-inactivated horse serum (Gibco BRL), supplemented with 6.5 mg/ml glucose, 0.15 mg/ml glutamine, 30 mM $NaHCO_3$, 100 U/ml penicillin, and 50 μg/ml streptomycin. The slices were maintained at 37°C in a humidified incubator with 95% air and 5% $CO_2$.

## NEURONAL CELL MIGRATION

In the brain, neurons usually originate in the ventricular zone, where their precursor cells proliferate. They can then migrate radially to other layers in the brain, or tangentially to other regions of the brain (Rakic, 1990; O'Rourke *et al.*, 1997). A disruption in neuronal migration during central nervous system development has been postulated as the mode of pathogenesis for many of disorders. Recently, the cell migration hypothesis has been proven for microcehpaly, lissencephaly, subcortical band heterotopia, periventricular nodular heterotopia and cerebral palsy (Tsutsui *et al.*, 1999; Lammens, 2000). Microcephaly, lissencephaly and polymicrogyria have been reported to sometimes be associated with human congenital CMV infection (Bale, 1984; Tsutsui *et al.*, 1997). In animal experiments, developmental retardation with microcephaly was observed in about 25% of offspring exposed to infection in utero (Li and Tsutsui, 2000). Disturbance of the neuronal migration and loss of neurons were observed in the brains of MCMV-infected developing mice *in vivo*, which were identified by immunohistochemical staining of viral antigen (Shinmura *et al.*, 1997b). Furthermore MCMV-infected and uninfected CNS stem/progenitor cells were transplanted into the neonatal brains. The reduced number of infected stem cells were engulfed into the subventricular zone and expressed GFAP, but did not migrate further, in contrast to the uninfected stem cells (Kosugi *et al.*, 2000).

To observe the migration mechanism of the CMV infected cells of the brain, we used the brain slice culture system. Roberts *et al* reported that in slice cultures from the neonatal ferret cortex, the initial migration of BrdU-labeled cells into the cortical plate was similar to that in cultured slices and normal animals for the first week. However after 1 week of culture, the labeled cells were distributed more widely than in vivo (Roberts *et al.*, 1993). By using the micromanipulator we have developed procedures for the infection of MCMV to the subventricular zone (SVZ) of brain slice cultures and showed that infected neurons migrate to the cortex in a similar way to that seen in in vivo infection. The infected neuronal cells expressing the early viral antigen migrated from the SVZ to the cerebral cortex, like infected neuronal cells in *in vivo*

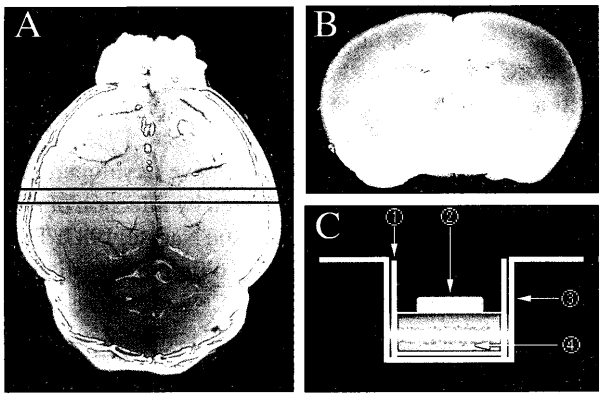


**Fig. 1** Preparation of the brain slice. **A:** The whole brains, dissected from 21-day old mouse. **B:** Brain slice of 400-μm thickness. **C:** Schematic representation of slice culture. (1) culture insert, (2) cerebral slice, (3) 6-well plate, (4) culture medium.

**Fig. 2** PI uptake of the hippocampus slice. The hippocampus slice was taken from 21-day old mouse and incubated for 7 days. A final concentration of 2 μM PI was added to the culture medium. The cellular uptakes of PI were recorded by fluorescence microscopy 3 h after the addition of the dye. CA1, CA2 and dentate gyrus (DG) regions had strong intensity of the PI uptake level.

(Shinmura *et al.*, 1997a), whereas the glial cells preferentially expressing the IE antigen remained in the infected spots detected by X-Gal staining (Shinmura *et al*, 1999). The neuronal migration of infected cells was not observed in the cerebral slices from 14- and 21-day-old mice. This system may reflect the infectious dynamics *in vivo*. With the combination of transgenic/knockout animals or functional blocking experiments (by adding drugs or antibodies to the slice) we could obtain more information for further understanding the mechanism of migration disorder of the brain.

## NEURONAL CELL DEATH

Neuronal degeneration or death plays an important role in the congenital anomaly of the brain. Apoptotic neurons and glia were detected in significant numbers in acute CMV encephalitis in humans (DeBias *et al.*, 2002). In our previous study, we showed quantitatively the neuronal cell loss occurred more in MCMV-infected brains compared with neuronal cells of uninfected brains (Shinmura *et al.*, 1997b). Interestingly apoptic cells were detected predominantly in non-viral-infected neuronal cells in in vivo CMV infections (Kosugi *et al.*, 1998).

In recent years, organotypic slice cultures have been used increasingly to study the neuronal death by hypoxia (Pringle *et al.*, 1997), hypoglycemia (Tasker *et al.*, 1992), or a combination of these to mimic ischemia (Breder *et al.*, 2000), excitotoxins (Kristensen *et al.*, 2001), other neurotoxis (Noer *et al.*, 2002), oxidative stress (Moskowitz *et al.*, 2001), and organic solvents including ethanol (Light *et al.*, 2002). In most of these studies, the cellular uptake of the fluorescent dye propidium iodide (PI) has been used as a marker for dying or degenerative cells (Noraberg *et al.*, 1999). **Fig. 2** shows the degeneration level of the hippocampus slice from a 21-day old mouse after 7 days of incubation. It shows the strong PI uptake in the CA1, CA2 and dentate gyrus regions. It is possible to measure the extent of neuronal injury and to identify the location of the vulnerability in the brain slices by reading the PI fluorescence staining level (Yin *et al.*, 2002). Making the brain slice itself causes injury and degeneration to the brain slices. To see the genuine degenerative effect of the virus, protein from the virus of interest should be transduced into the brain slice soon after preparation. This can be performed with new technology by making the fusion protein with pep-1 or TAT, and observe the neural degeneration chronologically (Schwarze *et al.*, 1999; Morris *et al.*, 2001).

Many attempts have been made to identify the signal transduction cascades that mediate nerve cell damage in the CNS (Wieloch *et al.*, 1996; Billingsley and Kincaid, 1997). Neuronal injury is associated with alterations in multiple signaling systems, including the protein kinase C cascades (Durkin *et al.*, 1997), the MAP kinase pathways (Bergmann *et al.*, 2002), the Ca2+/calmodulin-dependent protein kinase cascade (Ghosh and Greenberg, 1995), the nitric oxide signaling system (Strijbos *et al.*, 1996), mitochondoria toxins (Luetjens *et al.*, 2000), and caspase signaling system (Yuan and Yankner, 2000). Treatment with the inhibitors of these signaling pathways in the brain slice makes it possible to analyze the neuronal-death-pathway triggered. Ruden *et al* reported that MAP kinase inhibitor protected the brain slice from neural degeneration triggered by okadaic acid (Runden *et al.*, 1998). Brain slice culture is a powerful method to find neuroprotective drugs or treatment against many different conditions, including CMV, of the developing brain.

## SUSCEPTIBILITY OF THE CYTOMEGALOVIRUS TO THE BRAIN

The critical period of insult and the type of disorder mechanism can determine the phenotype of congenital anomaly of the brain. Investigation of the susceptibility of CMV to the brain from developmental viewpoints would help to elucidate the pathology and treatment of this disease. The susceptibility of mice to MCMV infection in vivo has been reported to diminish with age (Hayashi *et al.*, 1985; Tsutsui, 1995). The resistance is thought to be due to development of host defense mechanisms such as those mediated by natural killer (NK) cells (Brutkiewicz and Welsh, 1995) and macrophages (Booss *et al.*, 1989). Standardizing experimental conditions for *in vivo* analysis of brain susceptibility to viral infections is rather difficult. However, the susceptibility of cells to the CMV infection *in vivo* is markedly different from that *in vitro* (Britt and Alford, 1996). It should be noted that analysis of isolated cultured cells may not reflect the phenomena that occur in vivo in terms of in situ cellular interactions and defense responses. We have reported the effects of murine cytomegalovirus (MCMV) infection on the developing mouse brain in terms of susceptible cells and age-related resistance to MCMV in brain slice cultures (Shinmura *et al.*, 1999; Kawasaki *et al.*, 2002). **Fig. 3** shows that brain slice cultures from 0-day-old and 21-day-old BALB/c mice were infected with recombinant MCMV (RM461) ($5 \times 10^6$ PFU/ml) by whole immersion, and cultured for 3 (**Figs. 3A and 3C**) or 5 days (**Figs. 3B and 3D**). The brain slices were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside), and b-Gal (β-galactosidase) expression was visualized as blue spots. Staining of cells by X-Gal indicates expression of the late gene of MCMV in these cells. In the 0-day-old slices, virus-infected cells were scattered throughout the slices (**Figs. 3A and 3B**). In the 21-day-old slices, virus-infected cells tended to be localized in the marginal regions of the cortex and around the ventricles (**Figs. 3C and 3D**). The number of infected cells stained with X-Gal decreased with increasing age. Virus-infected cells were double-stained with antibodies to stem cell markers such as nestin (**Fig. 4B**) and Musashi-1 (**Fig. 4C**) (Mckay, 1997; Barres, 1999; Kaneko *et al.*, 2000) and also to GFAP (**Fig.4A**) (Doetsch *et al.*, 1999; Alvarez-Buylla *et al.*,

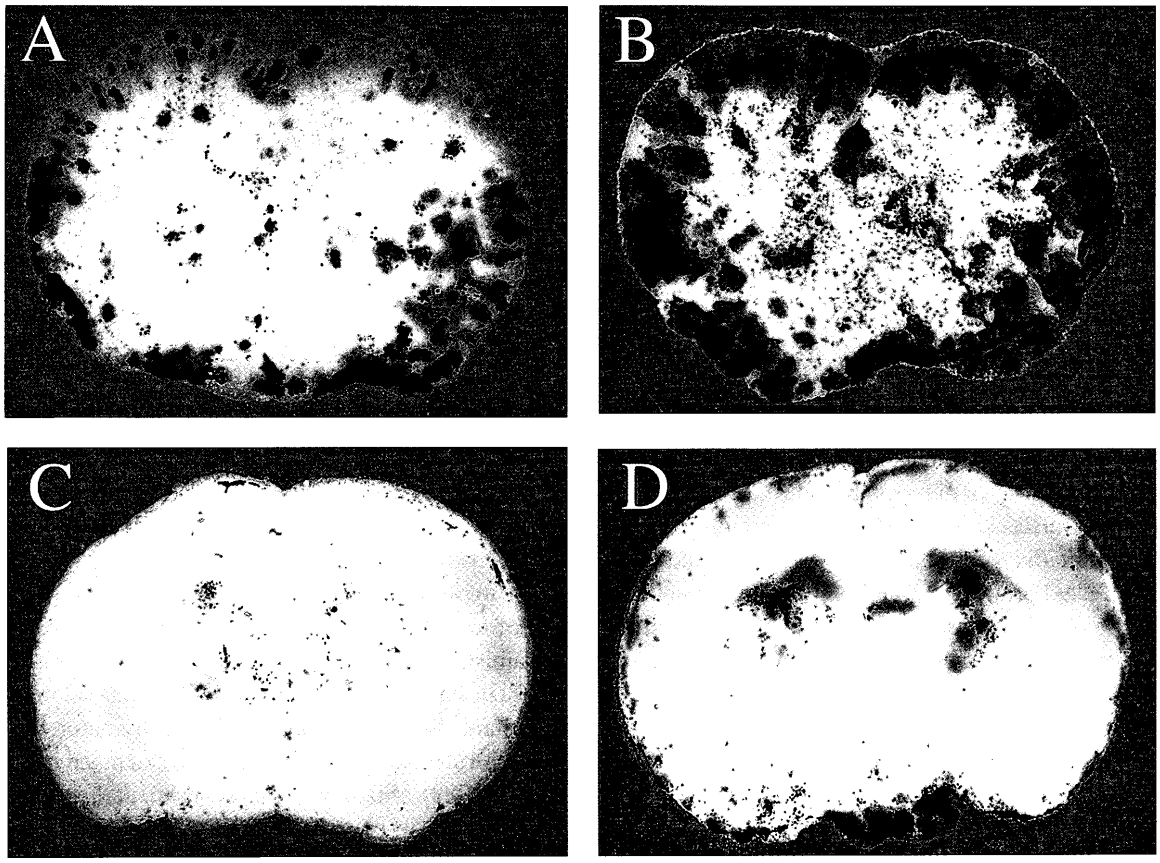


**Fig. 3** Age-dependent changes of β-Gal expression in the brain slice cultures from mice of different ages after MCMV infection. The brain slice cultures from 0-day-old (**A, B**) and 21-day-old (**C, D**) BALB/c mice were infected with recombinant MCMV (RM461) ($5\times10^6$ PFU/ml) by whole immersion, and cultured for 72 h (**A, C**) or 120 h (**B, D**). In the 0-day-old slices (**A, B**), virus-infected cells were scattered all over the slices. In the 21-day-old slices (**C, D**), virus-infected cells tended to be localized in the subventricular zone (SVZ) and the marginal regions of the cortex.

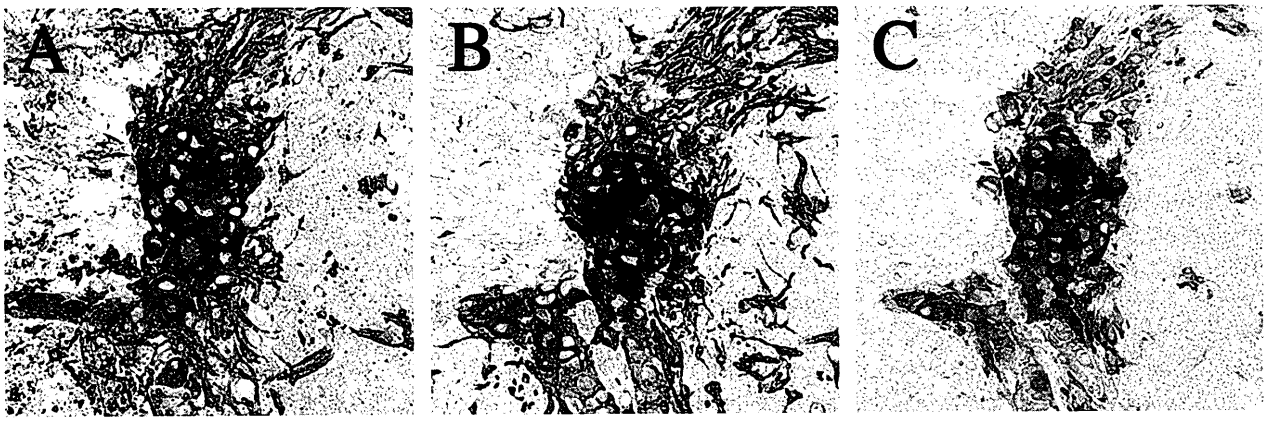


**Fig. 4** Immunohistochemical analysis of X-Gal-stained brain slices using antibodies to neural progenitor cell markers. Brain slice cultures from 21-day-old mice were infected with RM461 and stained with X-Gal 3 days after infection. The sections were immunohistochemically stained using antibodies to GFAP (**A**), nestin (**B**), Musashi-1 (**C**). X-Gal-positive cells (blue) around the subventricular zone (SVZ) were double-stained with antibodies to GFAP (**A**), nestin (**B**), Musashi-1 (**C**).

2001). Cells susceptible to MCMV infection in the SVZ were thought to be glial immature cells and neural progenitor cells, including neural stem cells. Kosugi *et al.* reported that MCMV infects EGF-responsive CNS stem/progenitor cells and inhibits the growth and DNA replication of stem/progenitor cell (Kosugi *et al.*, 2000). Immature glial cells, including neural progenitor cells, in the SVZ and marginal area were the most susceptible to MCMV in brain slice cultures from neonatal mice, and the susceptibility declined as the number of immature glial cells decreased with age. These results suggest that the number of the immature glial cells may be a primary factor of the susceptibility of the brain to CMV infection in the acute infection stage. The brain slice culture is a useful method to analyze the susceptibility of the viruses or number of insults to the brain during different developing stages.

## IMMUNOLOGLICAL ASPECT OF THE BRAIN SLICE CULTURE

The general view is that CMV is more damaging to the developing brain than to the mature brain because the systemic immune system is too immature during development to eliminate the CMV infection. The resistance is thought to be due to the development of the host's defense mechanisms such as those mediated by natural killer (NK) cells (Brutkiewicz and Welsh, 1995) and macrophages (Booss *et al.*, 1989). These cells have been shown to fight CMV infections and the efficacy of these systems increases with development. In spite of the fact that the innate immune system's activation during cytomegalovirus infection in the developing mouse brain (Kosugi *et al.*, 2002), there seems to be another aspect of the susceptibility of CMV to the brain. Recently van den Pol *et al* suggested that developing brain cells are inherently more susceptible to CMV infection, independent of the immune system. After intracerebral inoculation, the number of CMV-infected cells in neonatal brains was many times greater than in mature control or mature immunodepressed SCID mice, and the mortality rate of neonates was substantially greater than SCID or control adults (van den Pol *et al.*, 2002). In order to know whether immune responses are associated with susceptibility to MCMV infection, we used the brain slice culture system. We compared the susceptibility of brain slices from BALB/c mice with that of slices from Beige-SCID mice. This immunocompromised strain is deficient in both T and B cell functions and has reduced natural killer cell activity. Previously we reported that immature glial cells proliferated when brain slices were cultured for a prolonged time and were susceptible to CMV (Kawasaki *et al.*, 2002; Tsutusi *et al.*, 2002). Brain slices from 21-day-old BALB/c and Beige-SCID mice were infected with the mutant MCMV (RM461). X-Gal staining was performed 3 days after infection. Unexpectedly the amount of virus-infected cells stained with X-Gal was lower in the Beige-SCID mice than in the BALB/c mice. This tendency was also observed in the slices cultured for any period prior to infection (**Fig. 5**). The ratios of X-Gal-expressing area to whole-slice area of Beige-SCID mice were lower than those observed in BALB/c mice when compared quantitatively. For comparison of the amount of neural progenitor cells in the brain sections between Beige-SCID and BALB/c mice, we counted the cells immunostained with the antibodies to GFAP, nestin and Musashi-1 in 0-hour-cultures and 14-day-cultures prior to infection. The numbers of the cells stained for neural progenitor cell markers (GFAP, nestin and Musashi-1) were significantly lower in Beige-SCID mice than in BALB/c mice(data not shown). In this experimental model we confirmed that the number of the immature glial cells/ progenitor cells is a primary factor for susceptibility. The question remained why Beige-SCID mice showed lower proliferation rates of neural progenitor cells than normal strains of mice. Immunological factors such as cytokines might affect the proliferation of the neural progenitor cells in the brain slice. By observing reactivation in brain slice culture taken from 6-month-old mouse infected at the neonatal period, Tsutsui *et al.* proved that latent infection occurs in the mouse brain (Tsutsui *et al.*, 2002). These experimental models show the intermittent reactivation of CMV that occurs long after birth in individuals with congenital CMV infection or in immunocompromised adults in the central nervous system. The neural stem/progenitor cells may play an important role in this mechanism.

## GENE TRANSFER INTO THE BRAIN SLICES

In CMV infection, susceptibility to viral infection at the cellular level seems to be associated with transcriptional regulation rather than with viral receptors. The receptor for CMV has not been identified, although a few candidates have been reported (Nowlin *et al.*, 1991). The IE gene promoter of MCMV, the activation of which is dependent on cellular transcription factors that bind to the DNA sequence of the enhancer/promoter (Griffiths and Grundy, 1987; Mocarski, 1996), was transferred into brain slice cultures using a gene gun. Activation of the promoter was observed mainly in immature glial cells, including neural progenitor cells, of the SVZ. The number of cells in which the IE promoter was activated decreased with the brain development, whereas the number of cells able to activate the promoter transferred by gene gun was increased by culturing a slice for a longer preinoculation period (Kawasaki *et al.*, 2002). It is possible that the proliferation of cells, with the potential to activate this promoter, was associated differentiation stimulation of the susceptible cells from the stem/progenitor cells in the SVZ during the preinoculation period. This suggests that the susceptibility of neural cells to MCMV infections may be regulated at the level of transcription of the IE gene. In correlation with this, we

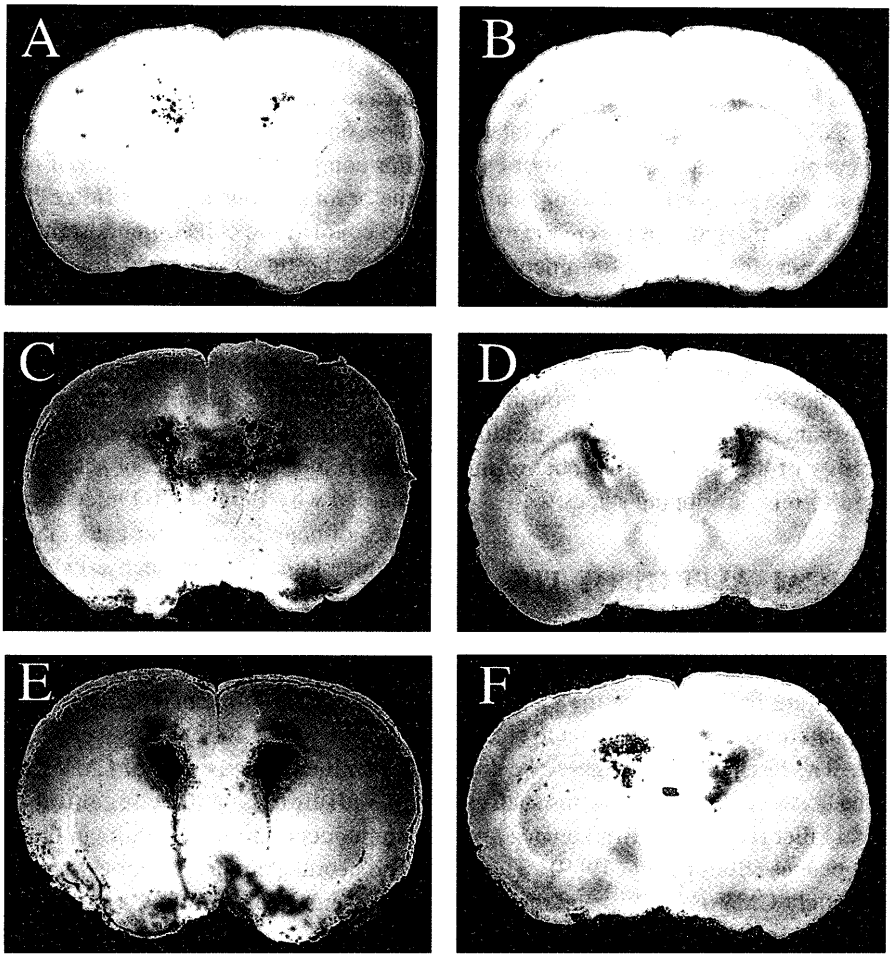


**Fig. 5** Increase of infected cells by prolonged culturing prior to MCMV infection. Brain slices from 21-day-old BALB/c mice (**A, C, E**) and Beige-SCID mice (**B, D, F**) were cultured for 0 day (**A, B**), 7 days (**C, D**), 14 days (**E, F**) prior to infection with mutant MCMV (RM461). The infected slices were incubated for 3 days, fixed and stained with X-Gal. The amount of X-Gal-positive cells increased when the culture period was longer prior to infection. The amount of virus-infected cells was lower in the Beige-SCID mice than that in BALB/c mice.

reported that the IE promoter was activated in glial progenitor cells in the SVZ during brain development in transgenic mice (Aiba-Masago *et al.*, 1999; Li *et al.*, 2001). In addition to the gene gun, any viral vectors can transfer a gene of interest into brain slice cultures. Ehrengruber *et al* characterized recombinant Semliki Forest virus (SFV), adenovirus type 5 (Ad5), adeno-associated virus type 2 (AAV), lentivirus, and measles virus (MV) by their expression of green fluorescent protein (GFP) in rat hippocampal slice cultures (Ehrengruber *et al.*, 2001). van den pol *et al.* investigated the possibility of cytomegalovirus as a useful vector for gene transfer to the brain (van den pol *et al.*, 2000).

## FOR FURTHER STUDY

The incidence of CMV-induced neurological problems has been estimated in 0.1% of births, with a possibility of subtle problems, such as learning deficits, in infected children. Synaptic roles for learning and behavioral disorders are being revealed. Synapses form the basic currency of information exchange between neurons and underlie the basic physiological function of circuits throughout the brain. In 1973 Bliss and Lomo by using brain slice made the remarkable discovery that major synaptic pathways in the hippocampus undergo a long-term form of synaptic plasticity (long-term potentiation, LTP) in response to a burst of high-frequency stimulation (Bliss and Lomo 1973). Patients who suffered from CMV brain infection with no morphological deformity of the CNS might have synaptic plasticity disorders causing learning disabilities or behavioral disorders. A brain slice culture using the electrophysiological method would be useful to reveal these disorders.

## CONCLUSION

Cytomegalovirus (CMV) is the most common infectious cause of congenital anomalies of the brain and also causes brain damage in immunocompromised individuals. Pathology of

CMV infection to the brain is highly associated with unique brain architecture, cell composition, migration and immune system. Studying CMV pathogenesis in the brain inevitably leads to the recent hot topics in the neuroscience world such as neural stem cell, migration, and apoptosis. Through CMV infection to the brain we can observe brain dynamic movements and functions. The brain slice system has characteristics more similar to *in vivo* than dissociated cells. The brain slice system is a useful method to investigate congenital and acquired CMV infection to the brain. Even though there are great advantages to using brain slice cultures, there are some disadvantages. Brain slice cultures are not a perfect model to represent *in vivo* phenomenon. Blood supply and natural immune responses seen *in vivo* are missing and culture-bias may considerably affect structural changes. The information and phenomenon obtained from the brain slice cultures should be compared with *in vivo* systems and dissociated cell culture systems.

Congenital anomaly of the brain might have started from observing the deformity of the brain. But recently the definition of congenital anomaly has been broadened to psychological and behavior disorders without particular morphological change of the brain. CMV may be one of the candidates for the cause of these disorders. Neural plasticity examined by electrophysiologically in the brain slice may play an important role in these areas. The brain slice culture system will hopefully give us new findings for congenital anomaly of the brain in the future.

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