cells when compared with that in transgenic mice with the HCMV IE promoter. Host cellular transcription factors may interact with the MCMV IE promoter more naturally in transgenic mice because of the homogeneity of the combination, although transcription factors are evolutionarily well preserved across the species. Alternatively, these observations may have been seen because the MCMV IE promoter region used was 1338 bp in length, far longer than those used for the HCMV IE promoter (−524/+13; and −67±54) [69,71]. It has been reported that the HCMV IE promoter may contain a modulator region located between nucleotides −750 and −1145 that negatively regulates the expression of the major IE genes in undifferentiated cells but positively influences the expression in differentiated cells [73,74]. It is possible that the MCMV IE promoter used contained such a region.

Activation of the MCMV IE promoter was analysed in the brain development of the transgenic mice [75]. During the early phase of neurogenesis, the transgene was expressed predominantly in endothelial cells, but not in neuroepithelial cells (Figure 5C, upper). During the later stage of gestation, expression of transgene was largely restricted to the ventricular zone (VZ) (Figure 5C, lower), similar to infected cells *in vivo* [20]. In neural precursor cells induced to differentiate from neurospheres of the transgenic embryo brains, expression of the transgene was detected in glial progenitor cells, expressing GFAP, nestin and Musahi, but not in cells expressing the markers of neuron (MAP2) and oligodendrocyte (myelin-associated glycoprotein: MAG) [75]. In postnatal development, persistent expression was observed in astrocyte-lineage cells. These spatiotemporal changes of MCMV IE promoter activity during brain development correlated with susceptible sites in congenital CMV infection in human [2,26].

Early e1 promoter in transgenic mice

In chronic infection of the developing brains in mice, the early nuclear antigen tends to be retained in neurons for a prolonged time after infection using antibody specific to the nuclear antigen (E1) [63]. This nuclear antigen was proved to be the product of MCMV early gene e1 (M112–113) [16] by immunostaining with the transfected cells, corresponding to HCMV early gene (UL112–113) from which nuclear DNA-binding phosphoprotein is expressed [76]. The products of the MCMV ie genes, especially ie3, were known to activate the early e1-promoter markedly by co-transfection experiments *in vitro* (Figure 5A) [77].

Transgenic mice were generated with an e1 enhancer/promoter region (1572 bp; −1534 to +38) connected to lacZ gene (MCMV e1-pro-lacZ) (Figure 6B) [78]. Surprisingly, expression of the transgene was completely restricted to the CNS in all three lines of transgenic mice (Figure 6D). The transgene was expressed in a subpopulation of neurons in the cerebral cortex (Figure 6D, right), hippocampus, diencephalon, brainstem, cerebrum and spinal cord. Non-neuronal cells in the CNS were negative for transgene expression.

It is known that the cascade of expression of the early genes of CMV is induced by the IE gene products together with host cellular factors [15,79]. The results suggest that the MCMV early e1 promoter can be activated in neurons of the CNS by some factors substituting for the IE products. Mocarski *et al.* reported that a deletion mutant of HCMV IE1 replicated under high infective titer conditions [80]. Therefore, it may not be necessary for the viral IE gene to be expressed for activation of the viral early expression in certain situations. Although the brain is the main target in congenital CMV infection and immunocompromised patients, no definite evidence that CMV has a special affinity for the CNS has been reported. The evidence that neuron-specific activation of the MCMV e1 promoter in the transgenic mice may account, at least in part, for the special affinity of CMV for the CNS. It has been reported that neurotropic virus infection in the CNS tends to become persistent in neurons [81]. The CMV IE promoters of both HCMV and MCMV are not well activated in neurons when compared with glial cells [82,83]. Therefore, the acute phase of CMV infection of the brain, in which lytic infection of glial cells is predominant, may convert to the chronic phase of infection in which persistent infection of neurons is predominant. Activation of the E1 gene may be important for transition of neurons to persistent infection.

POSSIBILITY OF PERSISTENT INFECTION IN NEURONAL CELLS

It has been reported that CMV causes ventriculoencephalitis in the developing brain in the acute phase of infection, and that infection then may

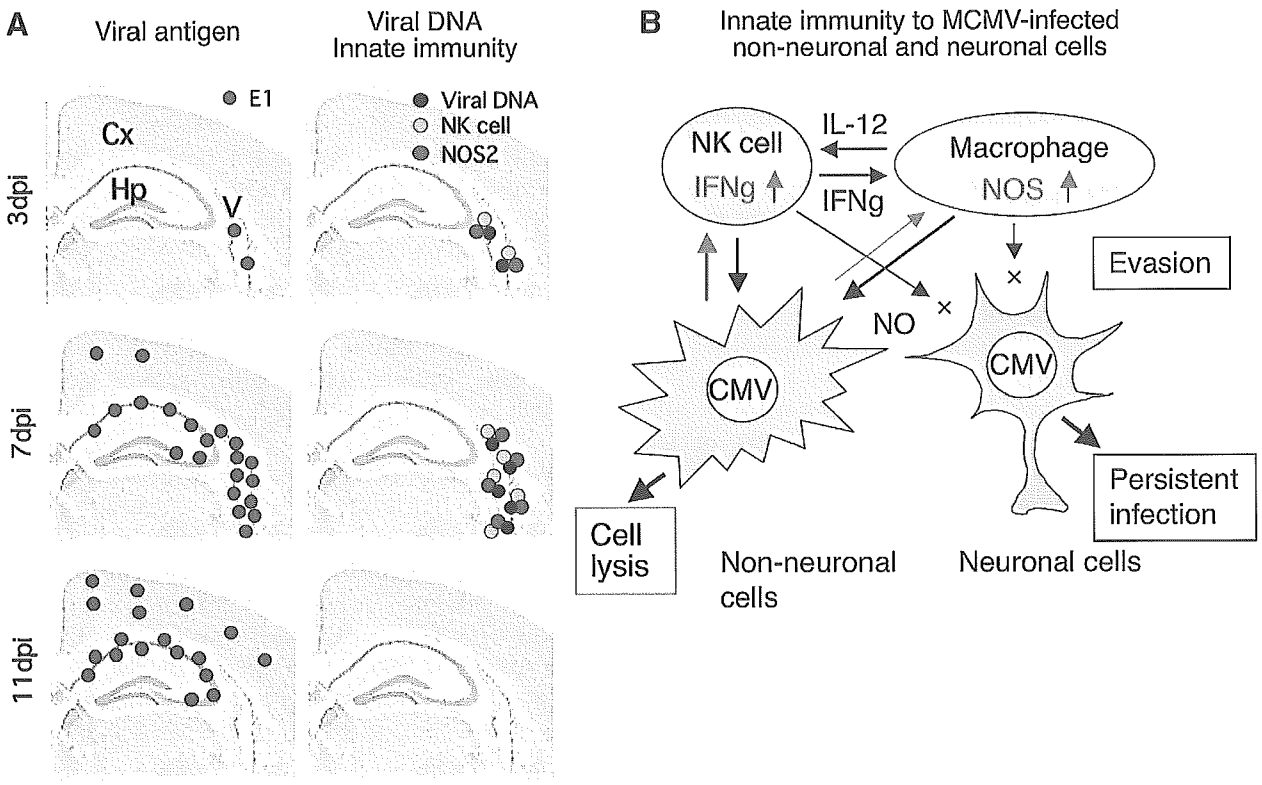

Figure 6. Innate immune responses to MCMV infection in the developing mouse brain and the evasion in the virus-infected neurons. (A) Schematic illustration of expression of the viral antigen. Viral DNA and innate immune reaction in the neonatal brains of newborn mice infected intracerebrally with MCMV. Comparison of cells in the ventricular walls (V) with pyramidal neuron of the hippocampus (Hp) in terms of viral antigen (E1) (blue), viral DNA (black), and NK cells stained with anti-aGM1 antibody (green) and NOS2-positive cells (red) at 3, 7 and 11 dpi. (B) Hypothetical schema concerning the difference in the innate immune response to MCMV-infected cells of non-neuronal cells in the lateral ventricle and MCMV-infected neurons in hippocampus

become chronic or persistent and result in functional neuronal disorders [9,26]. Similarly, MCMV infection in the developing mouse brain begins in the periventricular area and subsequently extends into the cerebral parenchyma and the E1 antigen tends to retain in neurons of the cortex and hippocampus [31,62]. We propose that acute infection in neurons of the developing brain transforms to persistent infection by evasion of innate immunity or anti-apoptotic effect in addition to the neuron-specific activation of the e1-promoter as described below.

Evasion of innate immunity

Coexistence of viruses with their hosts imposes an evolutionary pressure on both the virus and the host immune system. The host has developed an immune system able to attack the virus, and viruses have developed evasion mechanisms from this host immune surveillance [84]. Innate immunity produced by NK cells, macrophages and cytokines acts as a first line of host defense against viral infection. Subsequently, adaptive immunity works to eliminate virus via specific T cells and antibodies [85]. NK cells can secrete interferon (IFN)-γ, which not only has direct antiviral activity but can also act by stimulating the expression of nitric oxide synthase type 2 (NO2) in macrophages [86]. NOS2-derived NO appears to be important in the elimination of various viruses including CMV [87,88]. Furthermore, innate immunity seems to be primarily responsible for host defenses against CMV infection, especially during the perinatal period, because of the immaturity of adaptive immunity [89]. There have been several reports concerning the innate immune response during acute CMV infection in liver, spleen, lung, and salivary glands [88]. However, the role of innate immunity to CMV infection of the developing brain remains to be clarified.

From the aspect of defense mechanism, the brain is an immunologically privileged site which is separated from the immune system by the blood–brain barriers [90].

The role of innate immune responses caused by NK cells and NO derived from brain macrophages during MCMV infection in the developing brains of C57BL/6 mice was investigated [91]. The viral titer of the brains of neonatal mice inoculated with MCMV (half of LD_{50}) peaked at 7 days post-infection (dpi) then declined. Viral replication in the brain of newborn mice was significantly enhanced by administration of anti-asialo-GM1 antibody, a specific inhibitor of NK cells, or L-N6-(1-imminoethyl)-lysine, a specific inhibitor of NO2. Thus, NK cells and NO contribute to viral clearance from the brain. At early phases of infection (3 dpi), the MCMV E1 antigen-positive cells and viral DNA were detected in cells of the lateral ventricle (V). At 7 dpi the E1-positive cells were found not only in cells of the lateral ventricle but also in the neurons of the hippocampus (Hp) and cortex (Cx). At a prolonged phase of infection (11 dpi), the E1-positive cells disappeared from the ventricular wall, but persisted in neurons (Figure 6A). At 7 dpi, in the ventricular wall, aGM1 (NK cell marker), NOS (macrophage marker) were expressed markedly, whereas in the hippocampus, although the E1 antigen was expressed in neurons, the markers of NK cells and macrophages were hardly expressed. Viral DNA was also hardly detected in neurons of the hippocampus (Figure 6A, right).

It is hypothesised that some signals from the infected cells in the ventricular walls activate NK cells and macrophages. INFγ from NK cells may induce NOS in macrophages, then cytokines such as IL12 from the activated macrophages activate NK cells [86,87], resulting in lysis of the infected cells. In contrast, the prolonged infected neurons in the hippocampus may evade the innate immunity and transfer to persistent infection (Figure 6B). It has been reported that innate immune responses begin with the recognition of MCMV-infected cells by NK cells via the interaction between the Ly49H molecule of NK cells and the m157 protein of MCMV on virus-infected cells [92,93]. It is possible that expression of m157 proteins as ligands for Ly49 receptor of NK cells may be insufficient in MCMV-infected neurons to induce the innate immune response.

Concerning adaptive immunity to virus infected neurons, it was reported that neurons are deficient in major histocompatibility complex (MHC) class I molecules for presentation of viral antigens to specific cytotoxic T lymphocytes and suggested that virus infection would be persistent in these cells [94]. It has been clarified that MCMV encodes a set of genes for prevention or down-modulation of the MHC class I for the viral antigen presentation [95]. Combined with evasion of the innate and adaptive immune responses, virus-infected neurons may tend to become persistently infected cells.

Anti-apoptotic effect in neuronal cells

Generally, when a virus infects permissive cells, the virus replicates, then infective viruses are released for expansion of infection, while the infected cells are lysed. Virus-infected host cells express apoptotic genes to protect against viral replication and result in apoptosis by themselves. On the other hand, viruses express anti-apoptotic genes to survive, often in the form of persistent infection [96].

It was reported that MCMV infection preferentially persisted in cerebral neurons of the developing mouse brain [31], and that MCMV-infected neurons but not uninfected neurons are resistant to excitotoxic cell death induced by excess glutamate in primary neuronal cultures [97]. Apoptosis was induced in the developing brains by MCMV infection in association with neuronal cell loss. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells in the MCMV-infected brains rarely expressed MCMV antigens. In addition, the distribution pattern of the apoptotic cells was different from that of the viral antigen-positive cells and viral DNA-positive cells. These findings suggest that MCMV infection in the developing brains may induce apoptosis predominantly in the non-virus-infected cells. In primary neuronal cultures prepared from embryonic mouse brains, MCMV infection prevented induction of apoptosis by serum deprivation or by glutamate treatment [97]. A similar phenomenon was also reported in a study of a mouse model of CMV retinitis [98]. Many viruses have been reported to evolve mechanisms that block the host apoptotic process, presumably to allow cell survival in stringent conditions for

the virus to grow, resulting in persistent infections [99].

Possible effect of MCMV infection on neuronal functions

It has been reported that many neurotropic viruses cause persistent neuronal infection without cytopathicity [81,100,101]. Although the exact mechanisms of the viral persistence in neurons are not known, these studies showed that the intracellular milieu of neurons seems to restrict the level of viral gene expression and replication compared with those in non-neuronal cells that are fully permissive for viral replication.

The N-methyl-D-aspartate (NMDA) subtype of glutamate receptors plays an important physiological role in synaptic plasticity and cognitive functions by mediating an influx of Ca^{2+} ions through channels gated by these receptors [102,103]. NMDA receptor subunit 1 (NMDA-R1) protein is an essential component of functional NMDA receptors and is widely expressed in the brain [104,105]. It is evident that selective inhibition of the synthesis of NMDA-R1 prevents neuronal cell death induced excitotoxic brain injuries [106]. The amount of NMDA receptors expressed on neurons seems to be an important factor in determining the susceptibility of neurons to excitotoxic cell death.

The effects of MCMV infection on the expression of NMDA receptors was investigated in the hippocampus neurons of neonatal mice and primary neuronal cultures [107]. It was found that MCMV infection inhibits the expression of NMDA-R1 in the hippocampus and primary neuronal culture. The reduction of NMDA receptor expression is likely due to the reduced expression of NMDA receptors in MCMV-infected neurons rather than due to neuronal cell loss, because of scarce presence of TUNEL-positive cells. The reduction of expression of the NMDA-R1 of the CA1 region was more vulnerable to MCMV infection when compared with neurons of the CA3 region [107].

Neuronal excitotoxicity mediated by NMDA receptors has been implicated in the pathogenesis of brain damage induced by lentivirus [108], measles virus [109] and sindbis virus infection [103]. In these studies, it is emphasised that overstimulation of NMDA receptors by excess glutamate or neurotoxins generates apoptotic or necrotic cell death in infected or uninfected neurons. In our studies, MCMV-infected neurons resist excitotoxic cell death by excess glutamate [107] and inhibition of expression of NMDA receptor, presumably influencing neuronal function, and eventually inducing brain dysfunction.

LATENCY OF MCMV INFECTION IN BRAIN

A primary CMV infection is followed by a life-long persistence of the virus in a latent state and reactivation may occur later in life. HCMV infection is usually subclinical in immunocompetent individuals, but the virus can cause fatal disease in immunocompromised patients [110]. Latent CMV infection is reported to be in myeloid cells [111], macrophages [112] or lung cells in animal models [113]. Although brain is one of the principal target organs in congenital CMV infection and in immunocompromised patients, little attention has been focused on latent infection and the recurrence of CMV in the brain.

It is hypothesised that the brain disorders occur after recurrent reactivation of the latent infected cells in the brain. In order to test this hypothesis, the reactivation of latent MCMV infection was investigated in mouse brains by transfer to brain slice cultures [114]. It was first shown that latent infection with MCMV occurs in the mouse brain by the procedures. Mutant MCMV (RM461) [38] were infected into perinatal mouse brains and young adult mouse brain of 6-week-old mice and fed for 180 days, enough time for latent infection, then the brains were transferred to brain slice culture, and then cultured up to 4 weeks (Figure 7A). Reactivated cells were detected in the brain slices cultured for 2 to 3 weeks by β-galactosidase (β-gal) staining (Figure 7B). The β-gal-positive cells and infected virus increased during the slice cultures (Figure 7C). Reactivation was observed in about 75% of mice infected during the neonatal period 6 months after infection. Viral replication was also detected by plaque assay. Unexpectedly, reactivation was also observed in 75% of mice infected as young adults, although the numbers of infected cells in the slices was significantly lower than in neonatally infected mice. Interestingly, virus-reactivated cells were observed mainly in immature neural cells in the ventricular walls, expressing markers of the neural stem/progenitor cells such as nestin and Musashi [114].

As reactivation occurred in the brains of mice infected as young adults, it is possible that latent

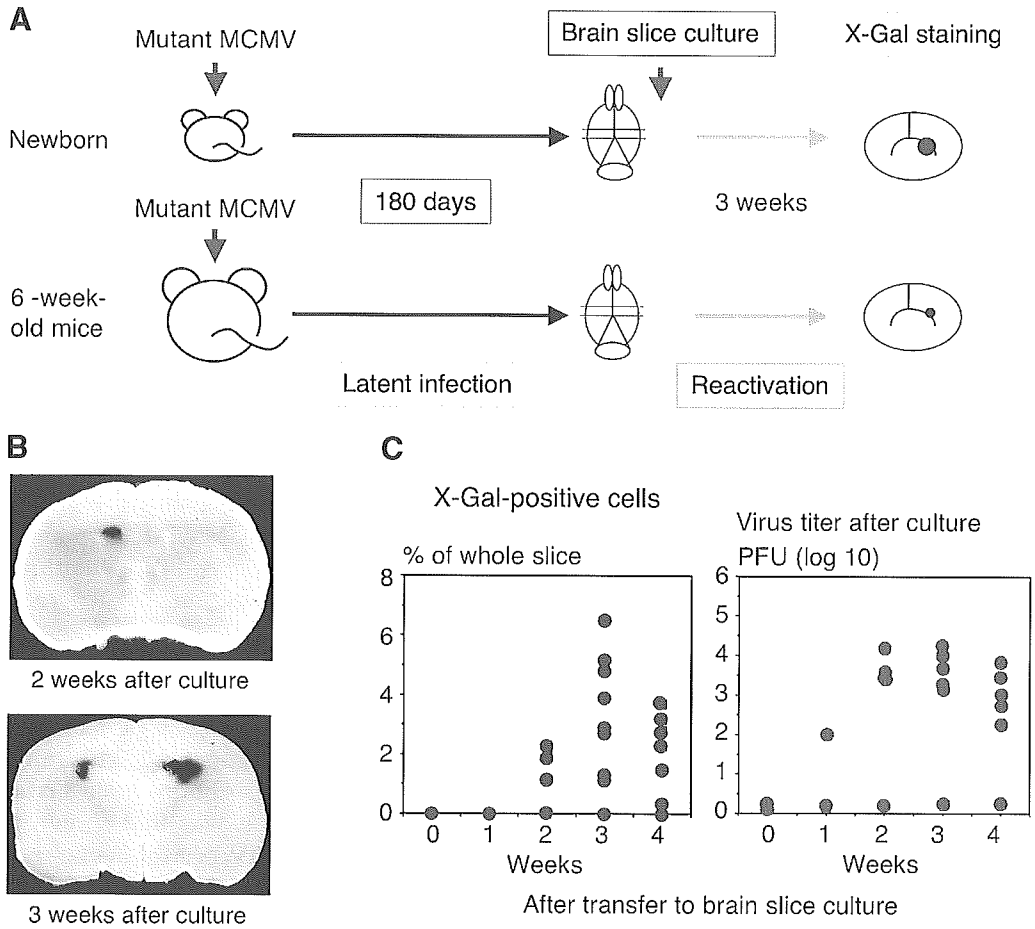

Figure 7. Reactivation of latent MCMV infection in mouse brain cells after transfer to brain slice culture. (A) For neonatal infection, BALB/c mice were injected intracerebrally with mutant MCMV (RM461) (5×10^2 PFU) within 24 h after birth. For young adult infection, 6-week-old mice were injected with RM461 (5×10^4 PFU). These two groups of mice were maintained for more than 6 months. For the uninfected control groups, neonatal and young adult mice were injected with MEM and maintained for the same period. Then, the brains were taken out and transferred to brain slice cultures until 4 weeks. (B) The slices were stained with X-Gal staining for β-galactosidase expression. (C) The proportion of stained viral infected cells in whole slices (left) and viral titers in slices (right) were measured every week

infection in the brains occurs not only in congenital infected individuals but also in immunocompromised adults. Although the mechanism of reactivation in the brain slice culture is not known, the basic mechanism may be similar to explant reactivation, in which latent cells are reactivated by co-culture of cell suspensions with susceptible fibroblasts [38,115,116]. It is not known whether there is a correlation between the susceptibility of neural progenitor cells to CMV infection and their tendency to be a preferential site of CMV latency. At this moment, it is difficult to distinguish neural stem cells from neural progenitor cells using neural markers. Neural progenitor cells are transient cells in the process of differentiation from neural stem cell to mature cells. It is not possible that these transient cells might be sites of latency. It is hypothesised that latent CMV infection may occur in neural stem cells. Once latently infected stem cells are committed to differentiate to progenitor cells by some kind of stimulation, latent virus is reactivated in these cells, in which a lot of factors for viral gene expression are induced, eventually leading to permissive infection. It is possible that neural stem cells might have strong suppressors for expression of the IE promoter [117] or might be deficient in factors necessary for activation of the promoter. At

present, it is difficult to demonstrate latent virus genome directly in neural stem cells, presumably because of the rarity of these cells and the difficulty in isolating these cells without induction of reactivation. Interestingly, our hypothesis that neural stem/progenitor cells may be the sites of latent infection in the brain may be related to the observation that hematopoietic progenitor cells are similarly sites of latent infection [111,118]. It was reported that reactivation occurred when these cells were stimulated to differentiate by certain factors, such as allogeneic stimulation [110,119].

CONCLUSION: IMPLICATION OF NEUROPATHOGENESIS IN CMV INFECTION

Early embryos, including ES cells, are not susceptible to MCMV. In contrast, embryonic brains are highly susceptible to MCMV infection (Figure 8, left upper). The cells responsible for the susceptibility are neural stem/progenitor cells in the ventricular walls. During differentiation, acute infection tends to occur in immature glial cells, consequently the lytic infection may cause disorders of brain development (Figure 8, right upper). CMV also infects neuronal cells, although lytic infection is difficult to observe. The possibility of persistent MCMV infection in cerebral neurons is indicated by the following specific neuronal features of MCMV infection: prolonged expression of the viral antigen and very low production of infectious virus, evasion of innate immune responses mediated by natural killer cells and macrophages, and decrease in the susceptibility of MCMV-infected neurons to excitotoxic cell death, in addition to neuron-specific activation of the MCMV e1-promoter. The possible persistent infection in neurons may influence neuronal functions, resulting in neuronal disorders (Figure 8, right lower). Human infants

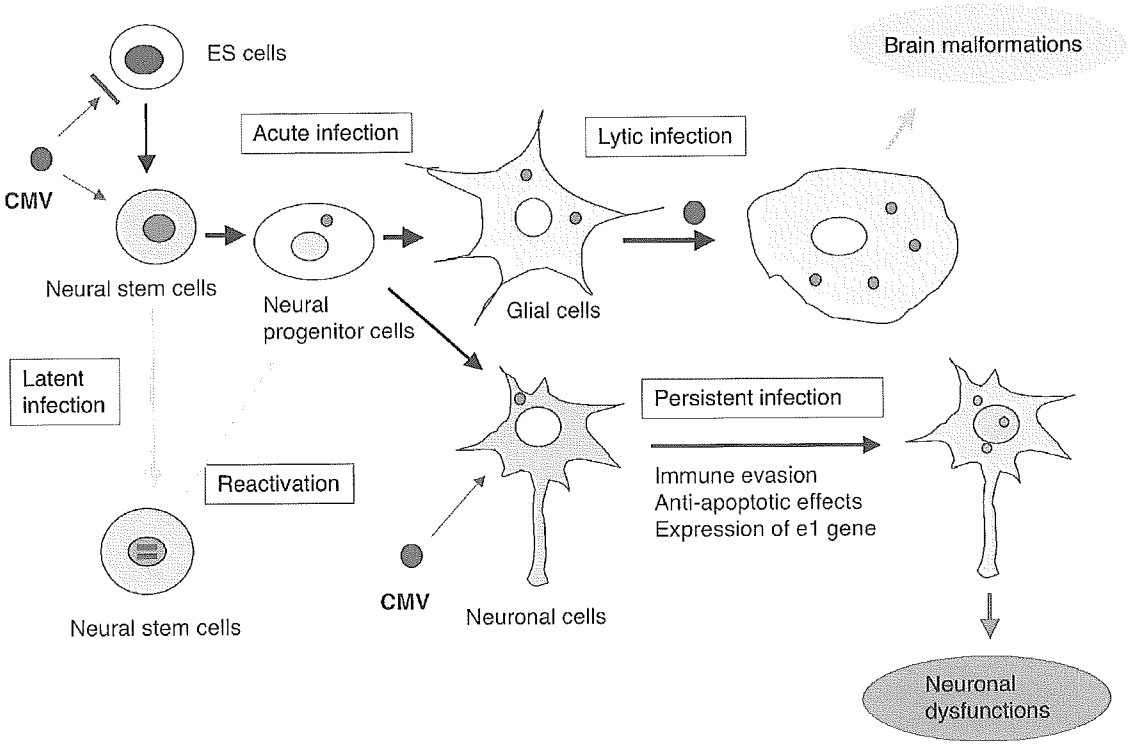

Figure 8. Schematic illustration of hypothetical neuropathogenesis of the brain disorders caused by CMV infection in the developing brains. In the early embryonic stage, ES cells are not susceptible to MCMV, but neural stem/progenitor cells in the embryonic brains are highly susceptible to CMV infection. In acute infection, immature glial cells undergo lytic infection, resulting in brain malformation. Infection of neuronal cells with CMV tends become persistent by evasion of innate immunity, anti-apoptotic effects, and neuron-specific activation of the e1 gene, causing neuronal dysfunctions. On the other hand, infection of embryonic brains with CMV may become latent in neural stem cells. After a long time period, the latent infection may be reactivated and become lytic in neural progenitor cells or glial cells

with symptomatic congenital CMV infection at birth frequently develop severe developmental disorders such as microcephaly, while infants with subclinical congenital infection subsequently have functional brain disorders such as mental retardation. The different infectious dynamics in the developing mouse brains may offer explanations for the pathogenesis of two conditions of congenital CMV infection in humans. On the other hand, CMV infection in the developing brain may become latent in neural stem cells. After a long time period, the latent infection may be reactivated by some stimulation such as immune suppression and lytic infection occurs in neural progenitor cells or glial cells (Figure 8, left lower), causing ventricular encephalitis or neuronal dysfunction by affecting the expression of neuronal genes such as NMDA receptor.

In perspective, it may be one of the most attractive subjects to study the mechanisms by which latent viruses in the brain affect the behavior and thought processes of human beings.

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Analysis of Human Cytomegalovirus UL144 Variability in Low-Passage Clinical Isolates in Japan

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Key Words
Cytomegalovirus · Genotypes · UL144 · gB · Congenital infection

Abstract
To explore a possible role for viral genes as determinants of virulence, portions of the UL144 tumor necrosis factor-like receptor gene and the UL55 envelope glycoprotein B gene from 42 patients with congenital human cytomegalovirus (HCMV) infection or other diseases were sequenced. Of the 42 patients, 16 (38%) had UL144 group 1 [group 1A, 15 of 16 (94%); group 1B, 1 of 16 (6%); group 1C, 0 of 16 (0%)], 5 patients (12%) had UL144 group 2, and 21 patients (50%) had UL144 group 3. Although group 1C was not found in Japan strains (0%), it was found in USA strains (22%). Other HCMV polymorphisms should be further evaluated for their potential relevance to neonatal infection, and acquired immunodeficiency syndrome-associated HCMV diseases.

Introduction

Human cytomegalovirus (HCMV) is a widespread human pathogen that has a minor clinical impact on healthy individuals, but causes various organ diseases in immunosuppressed patients and neural damage in fetuses infected in utero [1], and presents as a lifelong latent infection. However, latently infected HCMV is frequently activated in immunocompromised individuals such as patients with AIDS or organ and bone marrow transplants, thereby causing severe morbidity and eventual mortality [2]. However, the mechanisms of the viral pathogenesis have not yet been well understood.

The diversity of organs and cell types infected by HCMV in vivo has led to the hypothesis that HCMV disease and tissue tropism may be related to sequence variations among strains [3, 4]. Strain-dependent biological behavior depending on genetic polymorphisms has been examined most frequently in the region encoding UL55 envelope glycoprotein B (gB) [5], which was found to have a high level of sequence variability among clinical isolates [4, 6, 7]. gB is essential for HCMV infection both in vivo and in vitro. It is a target of neutralizing antibodies and mediates direct cell-to-cell transmission as well as virus entry from the extracellular environment [8]. A number of studies have attempted to reveal correlations of gB types

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with specific disease manifestations or sites of infection [4, 5, 7, 9]. However, a definitive association has not been established between gB types and HCMV diseases, leaving open the possibility that other variant HCMV-encoded products may play a role in viral pathogenesis, possibly in combination with gB.

HCMV pathogenesis has been investigated using well-characterized laboratory strains such as AD169 and Towne. These strains were derived from clinical isolates [10, 11], but have been extensively passaged in in vitro cell culture. A lower passage clinical strain, Toledo, was found to have a set of nineteen new open reading frames (ORFs), which were not present in the laboratory strains, but were present in several other low-passage clinical isolates [12]. Thus, the products encoded by these ORFs cannot be essential for HCMV replication in cell culture. However, the fact that the clinical isolates still retain these ORFs suggests that the predicted gene products may be essential for viral infection in vivo.

The possibility that these new ORFs may provide genetic markers for HCMV pathogenesis prompted us to investigate these genes in HCMV clinical isolates. Lurain et al. [13] analyzed the sequence variability of one of these ORFs, UL144, which encodes a protein homologous to the herpes simplex virus entry mediator [14]. Moreover, the UL144 ORF is a prime candidate for a pathogenesis marker because it is a member of the tumor necrosis factor receptor superfamily [15, 16]. Tumor necrosis factor receptor superfamily members play several roles in natural defense and adaptive immune responses that are critical for defense against viral pathogens such as HCMV [17].

In this report, we describe the significant strain-specific sequence variability for UL144 and gB in a large group of clinical isolates in Japan.

Materials and Methods

Cells and Specimens

All virus strains were propagated in human embryonic lung fibroblasts (HEL) in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Z. L. Bocknek Lab, Ontario, Canada), kanamycin (60 μg/ml), and 0.12% $NaHCO_3$. Forty-two HCMV clinical strains were isolated from 26 children with congenital infections (16 children in Miyazaki Prefecture, 8 in Kanazawa Prefecture, and 2 in Kagoshima Prefecture) and 16 adults recruited after organ transplantation or with other diseases (4 adults in Miyazaki Prefecture, 7 in Kanazawa Prefecture, and 5 in Kagoshima Prefecture).

Virus Isolation

Urine and blood samples and a throat swab were collected from the patients for HCMV isolation between 1989 and 1999. An informed consent was obtained from each patient prior to the sample collection. HCMV clinical strains were isolated by inoculating the fresh samples onto confluent monolayers of human embryonic lung fibroblast in 24-well plates (IWAKI, Scitech Div, Asahi Techno Glass, Chiba, Japan). Viral isolation was confirmed by detecting the specific cytopathic effect characteristic of HCMV. All the clinical HCMV strains were stored in liquid nitrogen as infected cells until use.

PCR Amplification

The clinical isolates were passaged in vitro less than five times prior to DNA extraction. Viral DNA was prepared from the infected cells by Hirt's method [18]. DNA samples were diluted in sterile distilled water to optimal concentrations for use as templates and stored at 4° until use. Two regions of the HCMV genome, gB and UL144, were analyzed using individual PCR primers for each. A 410-bp fragment encompassing the proteolytic cleavage site (nucleotides 1072–1482) of the HCMV gB gene (EMBL Accession No. X04606) was amplified with the following primers: forward, 5′-TCCGAAGCCGAAGACTCGTA-3′ and reverse, 5′-GATGTAACCGCGCAACGTGT-3′. The UL144 region was amplified using the UL144-B primer set described by Lurain et al. [13]. This primer pair, forward 5′-TCGTATTACAAACCGCGGAGAGGAT-3′ and reverse 5′-ACTCAGACACGGTTCCGTAA-3′, yield a 737-bp product corresponding to the complete coding sequence of UL144. Amplification was carried out with a programmable temperature control system, PC-700 (Astec Co. Fukuoka, Japan). The conditions for amplification with all primer sets were 94° for 5 min followed by 35 cycles of 94° for 1 min, 55° for 1 min, and 72° for 1 min. The 35 cycles were followed by a single extension at 72° for 7 min.

The PCR products were subjected to electrophoresis in 2% agarose gels. For the DNA sequencing, the amplified DNA was purified from the agarose gels using a QIAquik PCR purification kit (QIAGEN, Valencia, Calif., USA) according to the manufacturer's instructions.

DNA Sequencing

The purified PCR products were sequenced directly using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan, Tokyo). Sequencing reactions were performed at 96° for 10 s, 50° for 5 s, and 60° for 4 min for a total of 30 cycles. The sequencing products were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Amino acid sequences were derived from the UL144 DNA sequence data using a web-based translation program (URL: www.ebi.ac.uk/translate).

Phylogenetic Analysis

Sequence alignment was accomplished using the web-based Clustal W alignment programs (www/ebi.ac.uk/clustalW). Unrooted phylogenetic trees were constructed for the UL144 DNA sequence data using the above website (www/ebi.ac.uk/clustalW). The final tree was rendered with Tree View [19]. Amino acid sequences were aligned with a web-based translation program (URL: www.ebi.ac.uk/translate). Protein homolog of the predicted UL144 product were identified by a BLAST search of protein databases [20].

Nucleotide Sequence Accession Numbers

The UL144 sequences of the clinical strains have been assigned GenBank Accession No. AF084976–AF085005 and AF179196–AF179210.

Results and Discussion

Profiles of Clinical Strains

Cha et al. [12] suggested that the nineteen newly described ORFs could be responsible for HCMV virulence and tissue tropism, based on the Toledo strain and only five additional clinical isolates. However, it is necessary to demonstrate that these ORFs are consistently present in a large number of strains, such as our 42 clinical HCMV isolates (table 1). To confirm whether Japanese clinical isolates have nineteen ORFs as reported by Cha et al. [12], we examined UL144 gene variability of 42 clinical HCMV isolates in Japan.

A total of 42 clinical HCMV strains were collected from three different prefectures, Miyazaki (M series), Kagoshima (KG series) and Kanazawa (KS series), over the past 10 years. The clinical symptoms or clinical conditions were as follows: 4 patients with CMV retinitis, 2 with CMV skin disease, 4 with CMV pneumonitis, 3 with a kidney transplant, 3 with cancer, one with Hirschsprung disease, one pregnant woman, and 24 with congenital infection. The sample source of each strain is also listed in table 1. Since both UL55 (gB) and UL144 genes are polymorphic among HCMV strains [6, 13, 21–23], both of these genes were sequenced and compared among the 42 clinical HCMV isolates in Japan.

UL144 Variability

All the HCMV isolates were successfully amplified with the UL144B primer set, sequenced, and analyzed phylogenetically (fig. 1, table 2). All the HCMV strains could be categorized according to the schema of Lurain et al. [13].

This schema segregates the strains into five major groups as follows: group 1, 16 of 42 (38.1%), group 2, 5 of 42 (11.9%); and group 3, 21 of 42 (50%). The group 1 strains were further divided into groups 1A, 15 of 16 (93.7%), 1B, 1 of 16 (6.3%) and 1C, 0 of 16 (0%), with 1A being most closely related to the Toledo HCMV strain. The nucleotide polymorphisms conferred substantial amino acid substitutions when compared with Toledo (fig. 2). Only five of the current strains had sequences similar to those of the Toledo strain.

We also compared the nucleotide sequence data and clade classification according to the geographic sources of the HCMV strains. Although modest differences, only for the inside of group 1 strains, in the percentages of isolates conforming to each of the five UL144 clades were observed, the overall distribution of the UL144 grouping differed substantially between the USA and Japan strains (table 2).

Table 1. HCMV clinical strains

No.	Strain	Clinical history	Source	UL144 group	gB type
1	M8	Skin disease	Urine	1A	1
2	M9	Skin disease	Urine	1A	1
3	M14	Congenital infection	Urine	1A	1
4	M15	Congenital infection	Urine	1A	3
5	M16	Congenital infection	Urine	1A	1
6	M18	Congenital infection	Urine	1A	1
7	M19	Retinitis	Urine	1A	3
8	KG1	Congenital infection	Urine	1A	1
9	KG2	Congenital infection	Urine	1A	1
10	KG4	Hirschsprung disease	Urine	1A	3
11	KG5	Pneumonitis	Throat swab	1A	3
12	KG7	Pneumonitis	Blood	1A	1
13	KS2	Carcinoma (fever)	Saliva	1A	1
14	KS4	Leukemia (fever)	Urine	1A	1
15	KS8	Congenital infection	Urine	1A	1
16	KS12	Kidney transplant	Urine	1B	1
17	M4	Congenital infection	Urine	2	3
18	M12	Congenital infection	Urine	2	3
19	KS1	Retinitis	Tears	2	1
20	KS9	Congenital infection	Urine	2	1
21	KS10	Congenital infection	Urine	2	1
22	M1	Congenital infection	Urine	3	3
23	M2	Congenital infection	Urine	3	1
24	M3	Congenital infection	Urine	3	1
25	M5	Congenital infection	Urine	3	1
26	M6	Congenital infection	Urine	3	1
27	M7	Congenital infection	Urine	3	1
28	M10	Congenital infection	Urine	3	1
29	M11	Congenital infection	Urine	3	1
30	M13	Congenital infection	Urine	3	1
31	M17	Congenital infection	Urine	3	1
32	M20	Retinitis-encephalitis	Urine	3	1
33	KG3	Pneumonitis	Urine	3	3
34	KG6	Pneumonitis	Throat swab	3	1
35	KS3	Congenital infection	Urine	3	1
36	KS5	Retinitis	Tears	3	1
37	KS11	Kidney transplant	Urine	3	1
38	KS12	Congenital infection	Urine	3	1
39	KS14	Congenital infection	Urine	3	3
40	KS15	Kidney transplant	Urine	3	1
41	KS16	Leukemia (fever)	Urine	3	1
42	KS17	Pregnant women	Urine	3	1

UL55 Variability

The sequence analysis of UL55 (gB) in the 42 samples obtained from 24 congenital CMV infections and other patients revealed no statistical difference between the two groups for the presence of the gB gene (tables 1, 3). Sequence analysis of UL55 (gB) in the 42 samples revealed that 33 of 42 (79%) were gB type 1 (Towne type)

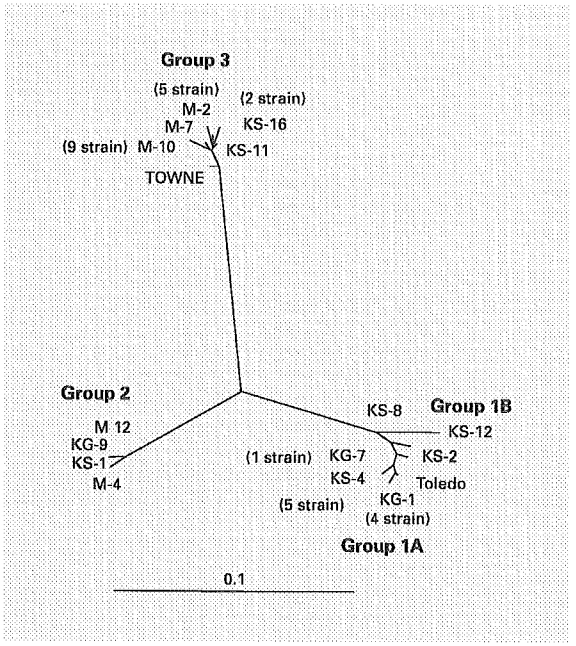
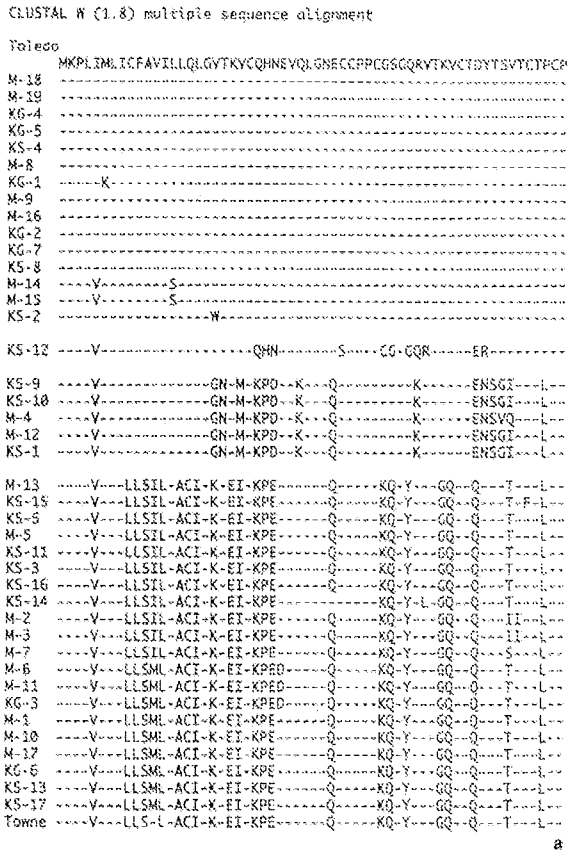


Fig. 1. Results of the phylogenetic analysis of the UL144 DNA sequences. An unrooted phylogenetic dendrogram showing the relationships of the HCMV strains according to the sequence polymorphisms of the UL144 gene. Group designations conform to the schema proposed by Lurain et al. [13]. Numbers indicate individual strains. M = Miyazaki strains; KS = Kanazawa strains; KG = Kagoshima strains.



a

Fig. 2a–c. Alignment of the amino acid sequences of the 42 clinical strains with the Toledo and Towne sequences as references. Numbers above the sequences represent amino acid residues. Hyphens indicate identity. Stars indicate deletions. Strains are listed according to sequence groups determined by phylogenetic analysis of the DNA sequences.

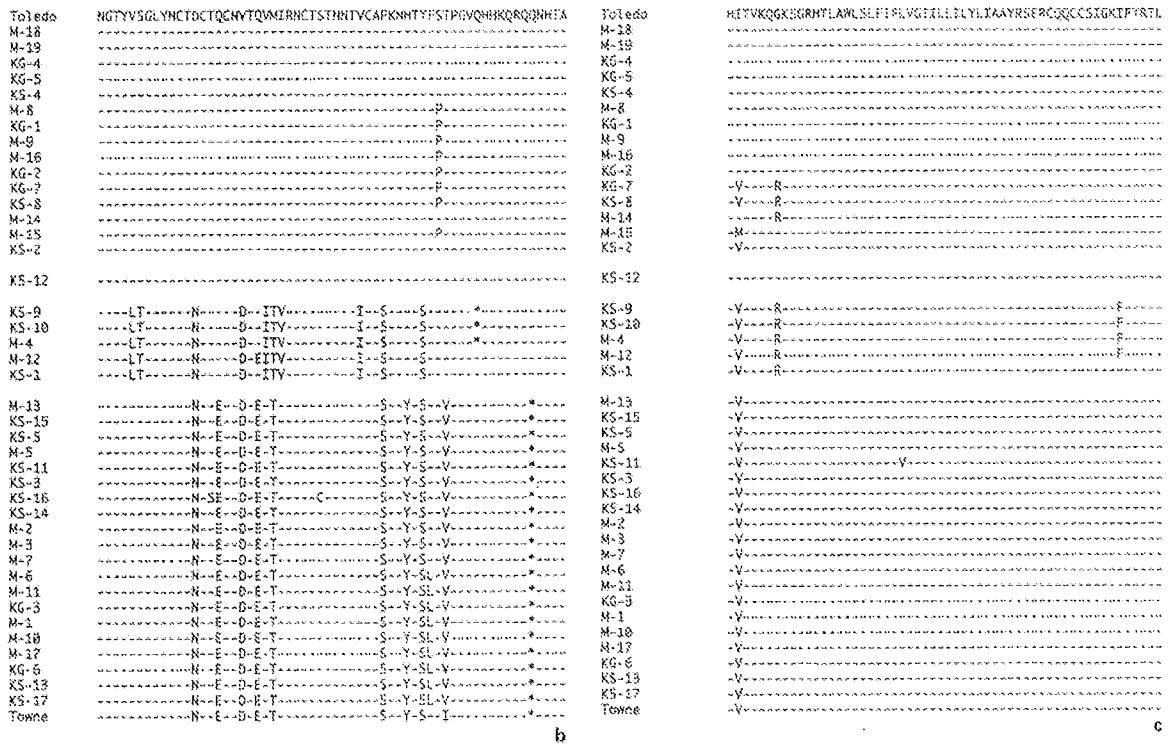
Table 2. Comparison of UL144 typing of clinical CMV isolates

	Group 1	Group 2	Group 3	Total
USA [12]	18 (40%) 1A 10 (56%) 1B 4 (22%) 1C 4 (22%)	4 (9%)	23 (51%)	45
Japan	16 (38%) 1A 15 (94%) 1B 1 (6%) 1C 0 (0%)	5 (12%)	21 (50%)	42

USA = Chicago, Maywood; Japan = Kanazawa, Miyazaki, Kagoshima.

Table 3. Comparison of gB typing of clinical CMV isolates

	Type 1	Type 2	Type 3	Type 4	Total
Wada et al. [24]	4 (20%)	15 (75%)	1 (5%)	0	20
Our data	33 (79%)	0	9 (21%)	0	42
Congenital	19 (58%)	5 (56%)			
Other	14 (42%)	4 (44%)			



and 9 (21%) were type 3 (table 3). None of the samples were types 2 or 4. In addition, no specific correlation of UL144 with the gB type was found statistically.

The Toledo and Davis laboratory-adapted strains showed gB type 3 and type 1 genotypes, respectively. On the other hand, Wada et al. [24] reported that, among 20 Japanese bone marrow transplant recipients, 20% had gB type 1, 75% had type 2, and 5% had type 3. This discrepancy is possibly due to a regional difference in the source of the patients who were analyzed.

One gene variation may contribute to differences in HCMV pathogenicity depending on the function of its product. In the case of HCMV, a gB polymorphism has been correlated with cell tropism and virulence [25]. In conclusion, precise definition of the genotypes of single viral proteins present in clinical strains, their immunological properties and their role in virus cell tropism might be extremely useful for understanding the virus pathogenicity. The comparison between UL144 and gB variability shown in this study suggests that gB and UL144 sequence clusters are not phylogenetically linked, as has been reported recently for the UL144 and UL4 genotypes [9, 13, 22, 26].

There was no correlation between the UL144 or the UL55 (gB) type and the mortality from HCMV infection in our study with only a small number of patients. However, a large-scale study with multivariant analysis is necessary to determine whether or not the virulence of each UL144 or gB type is different.

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Higher prevalence of human herpesvirus 8 DNA sequence and specific IgG antibodies in patients with pemphigus in China

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Background: Environmental factors, including virus infection, may play a role in the onset and/or development of pemphigus. However, it is controversial whether human herpesvirus (HHV)-8 is involved in pathogenesis of pemphigus.

Objective: The possible association of pemphigus with HHV-8 was investigated.

Methods: A total of 36 lesional skin and 13 peripheral blood mononuclear cell specimens from 58 patients with pemphigus, and 18 normal skin and 230 peripheral blood mononuclear cell specimens from healthy individuals, were tested for HHV-8 DNA sequence by a nested polymerase chain reaction assay. In all, 29 sera from the patients and 109 sera from healthy individuals were tested for HHV-8-specific IgG antibodies by enzyme-linked immunosorbent assays using HHV-8-specific oligopeptides as antigens.

Results: Prevalence of both HHV-8 DNA sequence (36.1% and 30.8% in lesional skin and in peripheral blood mononuclear cells, respectively) and HHV-8-specific IgG antibodies (34.5%) for patients with pemphigus was statistically higher than that of control subjects (<8% in both assays). There was no significant difference in HHV-8 prevalence among different types of pemphigus.

Conclusion: HHV-8 infection might be a contributing factor in the development of pemphigus. (J Am Acad Dermatol 2005;52:460-7.)

Pemphigus is an autoimmune blistering skin disease mediated by autoantibodies against desmoglein 1 and 3. Genetic and environmental factors may play a role in its pathogenesis.[1] As genetic factors, HLA antigen class II, HLA-DR4, HLA-DR14, HLA-DQ1, and HLA-DQ3 were reported to be strongly associated with pemphigus vulgaris (PV),[2-7] and HLA-DR14 and HLA-DQ1 with pemphigus foliaceus (PF),[8,9] whereas HLA-DR17 contributed to resistance against PV and PF.[6,10] Moreover, HLA antigen alleles susceptible to pemphigus were different among ethnic groups.[2-15] Environmental factors, including thiol drugs, burn, UV and radiographs, virus infection, neoplasm, hormones, pregnancy, and nutritional and psychologic conditions, were also reported to trigger pemphigus.[16,17]

In 1974, Krain[18] first reported a pemphigus case accompanied with herpes simplex virus infection.

Abbreviations used:

ELISA:	enzyme-linked immunosorbent assay
HHV:	human herpesvirus
KS:	Kaposi's sarcoma
ORF:	open reading frame
PBMC:	peripheral blood mononuclear cell
PCR:	polymerase chain reaction
PF:	pemphigus foliaceus
PV:	pemphigus vulgaris

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Thereafter, several reports described pemphigus cases that were associated with infection of other herpesviruses, such as varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, and human herpesvirus (HHV)-6.[19-28] Some of these associations were confirmed by detection of viral DNA sequence in lesional skin biopsy specimens and/or in peripheral blood mononuclear cell (PBMC).[26-28] However, because herpesvirus infection was demonstrated only in a subset of patients with pemphigus, further studies should be performed to clarify herpesvirus involvement in the pathogenesis of pemphigus.

Historically, a high incidence of Kaposi's sarcoma (KS) and Castleman's disease was reported for patients with pemphigus.[29,30] In 1994, Chang et al[31] discovered a new member of HHV, HHV-8, in a patient with AIDS-associated KS. Subsequent studies demonstrated HHV-8 infection in patients with AIDS-associated, classic, endemic, and iatrogenic KS but rarely in healthy individuals, which proved the causal association of HHV-8 with KS. HHV-8 has also been detected in patients without KS such as those with primary effusion lymphoma and multicentric Castleman's disease.[32-34] Some researchers reported detection of HHV-8 DNA sequence in lesional skin from patients with PV and PF,[35-37] especially in perilesional vascular endothelial cells and basal keratinocytes.[36] They proposed a causal relationship of HHV-8 with pemphigus. However, it is still unclear whether HHV-8 plays any role in the pathogenesis of pemphigus, because other groups reported inconsistent results.[28,38-44]

To further investigate the possible association of pemphigus with HHV-8, we enrolled a larger number of patients than that of the earlier studies by others. We determined the prevalence of HHV-8 DNA in their cryopreserved lesional skin biopsy specimens and PBMCs and that of HHV-8-specific IgG antibodies in their sera.

METHODS

Specimens from patients with pemphigus

A total of 78 specimens, including 36 cryopreserved lesional skin biopsy specimens, 13 PBMCs, and 29 sera, were obtained from 58 patients with pemphigus (29 men and 29 women, age 21-78 years, mean age 50.1 years) with their informed consent (Table I). These cases comprised 37 PV, 1 pemphigus vegetans, 4 PF, and 16 pemphigus erythematous types. All patients were given a diagnosis by clinical and histopathologic findings and by direct immunofluorescence testing for intercellular deposits in lesional or nonlesional skin. Some patients were simultaneously tested for serum antibody against intercellular substance by indirect immunofluorescence on substrate of guinea pig esophagus or tongue. Among these patients, only 9 received oral prednisone (<80 mg/d) for less than 1 week before the specimens were taken, whereas the others did not receive any systemic immunosuppressive therapy.

Specimens from control subjects

In all, 18 normal skin specimens were obtained from excised skin tissues for plastic surgery (11 men and 7 women, age 13-40 years, mean age 38.1 years). In all, 230 samples of PBMCs and 109 sera were obtained from 230 healthy blood donors in our hospital (171 men and 59 women, age 19-54 years, mean age 34.6 years).

Nested polymerase chain reaction

Total genomic DNAs were extracted from skin biopsy specimens and PBMCs with a standard phenol-chloroform method. Concentration and purity of the extracted DNA were determined by a spectrophotometry (Spectronic, Rochester, NY).

Primer set KS1/KS2 and nested polymerase chain reaction (PCR) primer set NS1/NS2 were used to amplify a 233-base pair (bp) HHV-8 KS330Bam fragment and a 160-bp fragment, respectively, as previously described.[31,40] A 25 μL reaction system included 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 100 μg/mL bovine serum albumin, 0.2 mmol/L deoxyl nucleoside triphosphate, 0.625 U γ-Taq DNA polymerase (TaKaRa Biotechnology Dalian Co, Dalian, China), 0.1~0.25 μg template DNA, and 0.8 μmol/L of each primer. PCR amplification was performed in a T-Gradient thermalcycler (Biometra, Gottingen, Germany). Thermocycling conditions for the first round of PCR with KS1/KS2 primers were initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. One tenth (2.5 μL) of the PCR products were used as template in the second round of PCR with NS1/NS2 primers. Thermocycling conditions for the second round of PCR were the same as the first round. All amplified products were analyzed on 2% agarose gels. DNA extracted from a primary effusion lymphoma cell line (BC-1) that was HHV-8- and Epstein-Barr virus-positive[45] was used as a positive control. Primer set PC03/PC04 was used to amplify a 110-bp fragment of human β-globin for all samples to confirm the integrity of DNA samples and for lack of inhibitors in the amplification reactions.[46]

Restriction endonuclease analysis of PCR products

All 233-bp and 160-bp products were retrieved with DNA fragment recovery kit (TaKaRa

Table I. Patients with pemphigus

No./sex/age, y	Intercellular deposits on DIF	Anti-intercelluar substances on IIF	Type	HHV-8 DNA		Serum IgG	ISP therapy
				Lesional skin	PBMCs		
1/M/78	IgG	/	PV	–	/	/	×
2/M/37	IgG, IgA	/	PV	–	/	/	×
3/M/41	IgG	/	PV	+	/	/	×
4/M/52	IgG, C3	/	PF	–	/	/	×
5/M52	IgG, C3	/	PV	+	/	/	×
6/M/43	IgG, IgA, C3	/	PE	–	/	/	×
7/M/43	IgG, C3	/	PE	–	/	–	×
8/F/56	IgG, C3	1:64	PE	+	/	+	×
9/M/76	IgG, C3	1:64	PF	+	/	+	√
10/M/76	IgG, C3	–	PV	–	/	–	×
11/M/50	IgG	1:16	PV	–	/	+	√
12/F/60	IgG	/	PVe	+	/	/	×
13/F/53	IgG, C3	/	PV	–	/	/	×
14/F/40	IgG, C3	/	PV	–	/	/	×
15/F/55	IgG, C3	/	PE	–	/	/	×
16/F/40	IgG, C3	1:128	PE	–	/	/	×
17/M/44	IgG, C3	1:128	PV	+	/	+	×
18/F/67	IgG, C3	/	PV	+	/	/	×
19/F/67	IgG, C3	/	PV	–	/	/	×
20/M/62	IgG	/	PV	–	/	/	×
21/F/75	IgG, IgA, C3	/	PV	+	/	/	×
22/F/41	IgG	/	PE	+	/	+	×
23/F/58	IgG	/	PV	–	/	+	√
24/F/34	IgG	/	PF	–	/	/	×
25/M/73	IgG, C3	/	PE	+	/	/	×
26/F/45	IgG, C3	/	PV	–	–	/	×
27M/28	IgG	/	PV	–	–	–	×
28/M/56	IgG	/	PV	–	–	/	×
29/M/?	IgG, C3	/	PV	–	–	/	×
30/M/73	IgG, C3	/	PV	+	+	/	√
31/F/28	IgG	/	PV	–	–	/	√
32/M/25	IgG, C3	/	PF	–	–	–	×
33/M/58	IgG, IgA, C3	/	PV	+	/	/	×
34/F/36	IgG	/	PV	–	–	/	×
35/F/36	IgG	/	PE	+	+	/	×
36/M/37	IgG, C3	1:64	PV	–	–	/	×
37/F/46	IgG	/	PV	/	+	/	√
38/M/47	IgG	/	PV	/	–	/	×
39/M/60	IgG	/	PV	/	+	/	√
40/F/64	/	1:64	PE	/	/	–	×
41/F/30	/	1:1024	PF	/	/	+	×
42/F/56	/	1:256	PE	/	/	–	×
43/M/21	IgG	/	PV	/	/	–	×
44/F/?	/	1:256	PE	/	/	–	×
45/M/40	/	1:256	PV	/	/	–	×
46/M/59	IgG	1:64	PV	/	/	–	×
47/F/29	/	1:128	PE	/	/	–	×
48/F/57	–	1:80	PV	/	/	–	×
49/F/44	/	1:16	PV	/	/	–	×
50/F/53	/	1:128	PE	/	/	–	×
51/M/64	/	1:32	PV	/	/	+	√
52/M/60	/	1:32	PE	/	/	–	√
53/F/40	/	1:128	PE	/	/	–	×
54/F/49	/	1:32	PV	/	/	+	×