Table 1
HSV-1 replication in various types of human cell lines

Day after infection	TALL-1	CCRF-CEM	AKATA	THP-1	U937	FL
1	1.5×10^4	2.4×10^4	8.2×10^3	2.4×10^3	1.2×10^3	1.1×10^8
2	1.7×10^4	1.3×10^7	2.6×10^5	8.0×10^4	4.4×10^4	death
4	2.4×10^4	death	5.4×10^6	2.6×10^5	2.8×10^4	
6	2.8×10^4		5.0×10^4	2.0×10^5	3.6×10^4	

HSV-1 infections were at an MOI of 5. Culture medium was changed everyday. Virus titer in the collected culture supernatant was determined by a plaque-forming assay using Vero cell as an indicator.

Furthermore, we observed that virus replication in U937 and THP-1, which did not show SOCS3 induction by HSV-1 infection, did not interfere by the addition of WHI-P131 (data not shown).

Neutralizing anti-IFN-α and anti-IFN-β antibodies were added to cell cultures during HSV-1 infection in the presence of WHI-P131. The neutralizing antibodies highly and significantly released the inhibitory activity of the Jak3 inhibitor WHI-P131 on viral replication (Fig. 7). This suggests that the suppressed viral replication by the Jak3 inhibitor should be caused by autologously produced IFN. These results indicated that inhibition of the IFN system by virus-induced SOCS3 mainly contributes to efficient virus replication.

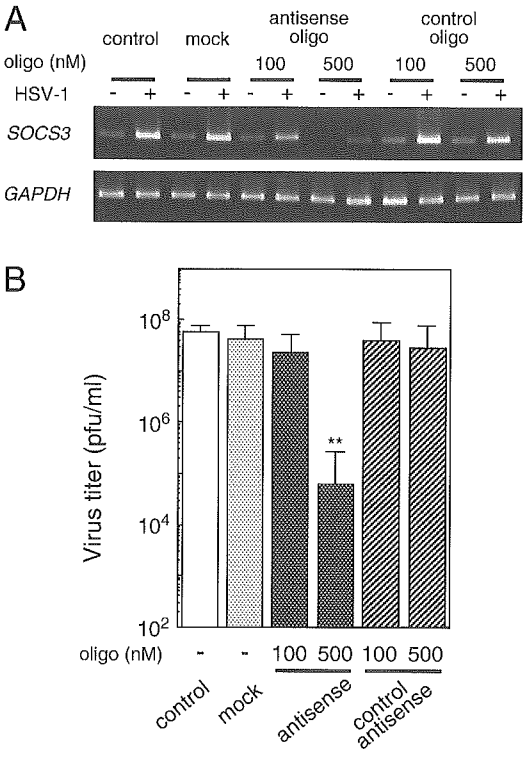

Fig. 4. Effect of SOCS3 antisense oligonucleotide on HSV-1 replication in FL cells. Phosphorothioate oligonucleotides of specific antisense for human SOCS3 and its mismatch control were transfected into FL cells. FL cell cultures were transfected three times at intervals of 36 h. Cultures were incubate for 48 h after the last transfection and then infected with HSV-1 VR3 at an MOI of 1. After 24 h of infection, virus titers in the culture medium were determined by the plaque-forming assay. Each experiment was carried out in triplicate and the data are present as mean ± SD. Mock: transfection with an unrelated plasmid. $^{**}P < 0.01$ compared to control.

Discussion

We previously reported that HSV-1 infection upregulates SOCS3, a host endogenous JAK/STAT inhibitor (Yokota et al., 2004b). SOCS family proteins have been shown to suppress various cytokine signaling through the JAK/STAT pathway by a negative feedback regulation. SOCS3 inhibits JAK phosphorylation, namely activation, by association with cytokine receptors (Alexander, 2002; Nicholson et al., 2000; Schmitz et al., 2000). To our knowledge, only two reports suggesting virus-induced SOCS3 have been published to date, our previous report (Yokota et al., 2004b) and a report described by Bode et al. (2003). Bode et al. demonstrated that human hepatitis C virus core protein transcriptionally induced SOCS3 that suppresses the IFN-induced anti-viral state. On the other hand, some IFN-resistant leukemia and lymphoma cell lines constitutively express SOCS3 (Brender et al., 2001; Sakai et al., 2002). Expression of SOCS3 coincided with constitutive phosphorylation of STAT3 in these cell lines. These lines of

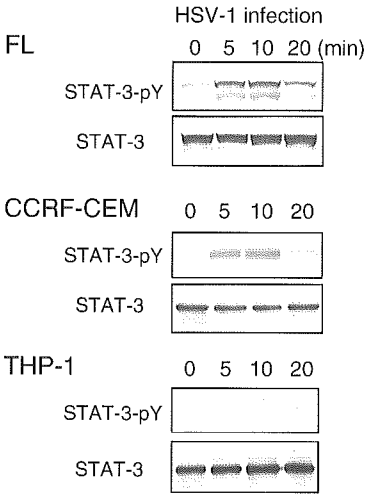

Fig. 5. Activation of STAT3 by HSV-1 infection. FL cells, CCRF-CEM cells and THP-1 cells were infected with HSV-1 VR3 at an MOI of 5. At various times after infection, cells were lysed and assessed by Western blotting with anti-Tyr-phosphorylated STAT3 (STAT3-pY) and anti-STAT3 antibodies.

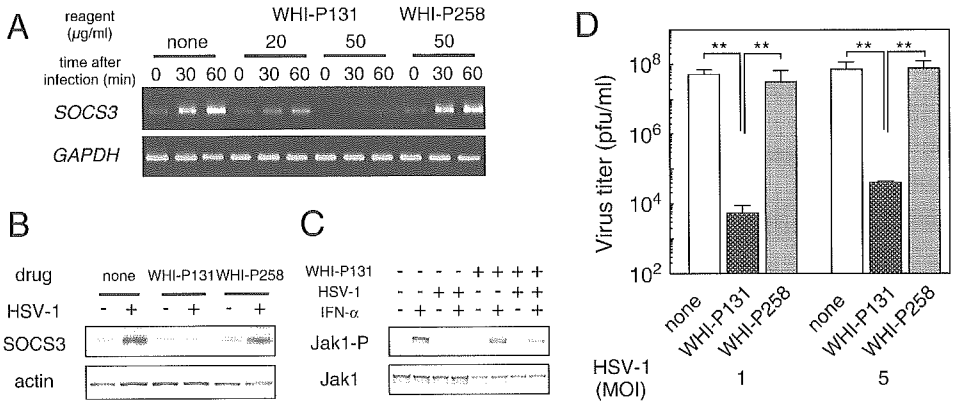

Fig. 6. Effect of the Jak3 inhibitor WHI-P131 on HSV-1 replication in FL cells. (A) Suppression of SOCS-3 mRNA induction by WHI-P131 determined by RT-PCR. FL cells were treated with 20 or 50 μg/ml Jak3 inhibitor WHI-P131 or its control WHI-P258 for 30 min. FL cells were then infected with HSV-1 VR3 at an MOI of 5 in the presence of WHI-P131 or WHI-P258. At various times after infection as indicated, total RNA was isolated. mRNA levels of SOCS3 were determined by semi-quantitative RT-PCR. GAPDH was carried out as a control. (B) Suppression of SOCS-3 protein induction by WHI-P131 determined by Western blotting. After 90 min of infection with HSV-1 VR3 (MOI 5), FL cells were lysed and assessed by Western blotting with anti-SOCS3 antibody. Actin was used as a control. (C) Effect of WHI-P131 on inhibition of IFN-α-induced Jak1 phosphorylation by HSV-1 infection. FL cells were treated with 50 μg/ml WHI-P131 for 30 min. Cells were then infected with HSV-1 VR3 at an MOI of 5 in the presence of WHI-P131. After 2 h of infection, cells were treated with 1000 IU/ml IFN-α for 15 min. Untreated controls for each step in the treatment were also examined. The cells were lysed and assessed Western blotting with anti-Tyr-phosphorylated Jak1 (Jak1-P) and anti-Jak1 antibodies. (D) Effect of WHI-P131 on HSV-1 replication in FL cells. FL cells were treated with 50 μg/ml WHI-P131 or WHI-P258 for 30 min. Cells were then infected with HSV-1 VR3 at an MOI of 1 or 5 in the presence of WHI-P131 or WHI-P258. After 24 h of infection, culture supernatants were collected and virus titer in the supernatants was determined by the plaque-forming assay. Each experiment was carried out in triplicate and the mean ± SD are presented. $^{**}P < 0.01$.

evidence indicate that SOCS3 is upregulated via activated STAT3 and it efficiently inhibits IFN-α/β signaling.

We found that cells (FL and CCRF-CEM) which showed strong induction of SOCS3 during HSV-1 infection become lytic infection with rapid HSV-1 replication. Cells (TALL-1) which showed weak SOCS3 induction and cells (U937, THP-1, and AKATA) which did not show any significant SOCS3 induction turned into persistent or prolonged infection. Among these cell lines, only TALL-1 did not show any cytopathic effects of HSV-1 and turned into a persistent infection. Low HSV-1 replication in TALL-1 might be caused by weak induction of SOCS3, but TALL-1 cells could have additional unknown resistance mechanisms against the cytopathic effects of HSV-1 and this remains to be investigated. These are consistent with our previous report describing the weaker induction of SOCS3 observed by infection with tegument protein (UL41 or UL13)-deficient mutant viruses compared with wild-type virus (Yokota et al., 2004b). UL41 and UL13 contribute to the hypersensitivity to IFN (Shibaki et al., 2001; Suzutani et al., 2000). The poor induction of SOCS3 by these mutants may be correlate with a lower replication efficiency (about one log lower virus titer) compared to the parental virus (data not shown). In order to confirm this relationship between SOCS3 inducibility and viral replication, we examined the effect of suppression of virus-induced SOCS3 on progeny virus production in FL cells. The results of transfection with antisense oligonucleotides specific for SOCS3 and treatment with the Jak3 inhibitor WHI-P131 both indicate that induction of SOCS3 is required for rapid replication of HSV-1 during the early infection phase in FL cells. SOCS3 contributes to suppression of signal transduction of many cytokines, including IFN, through a negative feedback mechanism (Alexander, 2002; Cooney, 2002; Krebs and Hilton, 2001; Yasukawa et al., 2000). Suppression of SOCS3 is expected to lead to transduction of IFN-α/β signaling by release of the negative feedback. To confirm the effect of SOCS3 on IFN-induced suppression of viral replication, we examined the effect of neutralizing anti-IFN-α and anti-IFN-β antibodies on suppressed viral replication

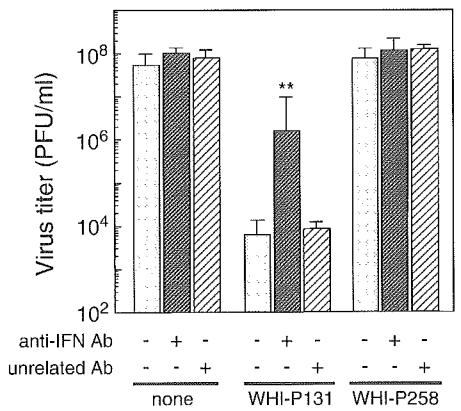

Fig. 7. Effect of neutralizing anti-IFN antibodies on inhibition of HSV-1 replication by the Jak3 inhibitor WHI-P131. FL cells were treated with anti-IFN-α and anti-IFN-β antibodies and 50 μg/ml WHI-P131 or WHI-P258 for 30 min. Cells were the infected with HSV-1 VR3 at an MOI of 1 in the presence of 50 μg/ml WHI-P131 or WHI-P258 and anti-IFN neutralizing antibodies. After 24 h of infection, culture supernatants were collected and virus titers in the supernatants were determined by the plaque-forming assay. Untreated controls for each step in the treatment were also examined. Each experiment was carried out in triplicate and the mean ± SD are presented. $^{**}P < 0.01$ compared to untreated control.

by the Jak3 inhibitor WHI-P-131. The neutralizing anti-IFN antibodies effectively released the suppression of viral replication (Fig. 7). The results indicate that HSV-1 replication is suppressed by autologously produced IFN in the absence of SOCS3. In some cell lines, HSV-1 is able to induce SOCS3 expression in order to suppress the antiviral activity of IFN. The neutralizing antibodies were only able to partially recover the levels of viral replication and this could be explained by the presence of other IFN species, such as ω and λ, or other cytokine systems regulated by SOCS3 which contribute to the SOCS3-mediated replication of the virus. These lines of evidence indicate that induction of SOCS3 is a key factor in rapid virus replication during acute infection. The major mechanism by which SOCS3 mediates rapid replication is through suppression of IFN-α/β signaling. Inducibility of SOCS3 varied among cell types, and this may be a deciding factor between lytic infection and persistent or prolonged infection.

Jak3 inhibitors have been examined for its potential in therapeutic use for cancer and graft versus host disease (Changelian et al., 2003; Malaviya et al., 1999; Sudbeck et al., 1999). Furthermore, it has been reported that SOCS3 promotes Th2 development by inhibiting IL-12-mediated STAT4 activation in T cells (Seki et al., 2003). Therefore, Jak3 inhibitors are also being considered to treat allergic diseases, such as asthma and atopic diseases (Wong and Leong, 2004). Now, we propose that Jak3 inhibitors have potentials of treatment for viral infection. It is an important point that SOCS3 is very rapidly induced by HSV-1 infection compared to other anti-IFN strategies such as disappearance of host proteins contributing to IFN production and signal transduction as described in Introduction. Jak3 inhibitors may suppress acute infection and rapid propagation of HSV-1.

Materials and methods

Cells and viruses

The human amnion cell line FL, the T lymphoblastoid cell line CCRF-CEM, and the monocytic cell lines U937 and THP-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The human T-cell leukemia cell line TALL-1 was obtained from the Japanese Collection of Research Bioresources (JCRB; Tokyo, Japan). AKATA (an EBV-negative clone) was described previously (Hariya et al., 1999). All cells were routinely cultured in RPMI-1640 containing 10% fetal bovine serum. The HSV-1 strain VR3 was obtained from ATCC. Unless otherwise mentioned, virus infection was performed at a multiplicity of infection of 5 (MOI 5). After 1 h of infection, culture medium was changed in order to remove residual virus in the culture medium. Virus titers in the culture supernatant were determined by a plaque-forming assay using Vero cells as an indicator.

Reagents

The Jak3 inhibitor (WHI-P131) and its negative control compound (WHI-P258) were purchased from Calbiochem (La Jolla, CA). The stock solutions of these compounds were prepared in dimethyl sulfoxide (DMSO) as a solvent. When the compounds were added to cultured cells, the corresponding amount of DMSO was added to control experiments. A sheep anti-leukocyte IFN-α antibody and a rabbit anti-IFN-β antibody were purchased from Chemicon (Temecula, CA) and Pepro Tech EC (London, United Kingdom), respectively. IFN-neutralizing culture using the neutralizing antibodies was performed as described previously (Yokota et al., 2004a). Briefly, 10 μl/ml of anti-IFN-α antiserum and 5 μg/ml of IFN-β monoclonal antibody were added to the culture medium. For control experiments, the same amount of an immunoglobulin G derived from normal sheep and rabbit sera were added to the culture media.

Transfection of antisense oligonucleotides

An antisense oligonucleotide specific for human SOCS3 was the phosphorothioate oligonucleotide 5′-GCAGCTGG-GTGACTTTCTCA-3′. This is the sequence used by Mahboubi et al. (2003). The mismatch control was the phosphorothioate oligonucleotide 5′-TGAGAAAGT-CAGGGAGCTGC-3′. To transfect FL cells with oligonucleotides, cells were cultured to about 30% confluency and then incubated with 100 or 500 nM phosphorothionate oligonucleotide and Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Cell cultures were transfected three times at intervals of 36 h. No significant cytotoxicity was observed in the transfection experiment. After an additional 48 h of culture, the cells were infected with HSV-1 at an MOI of 1. After 24 h of infection, the virus titer in the culture medium was determined by a plaque-forming assay.

Immunofluorescence microscopy

Adherent cells cultured on coverslips or floating cell smears were fixed with methanol at room temperature for 5 min, and then blocked with PBS containing 2% goat serum. The cells were stained with fluorescein-conjugated goat anti-HSV-1 antibody (Chemicon) and analyzed by fluorescence microscopy on an Olympus IX71 system (Olympus, Tokyo, Japan).

Western blotting

Preparation of total cell lysates, SDS-polyacrylamide gel electrophoresis, and Western blotting were carried out as described previously (Yokota et al., 1999, 2001). Rabbit anti-SOCS3 antibody was purchased from IBL (Gunma, Japan). Rabbit anti-STAT1 and was purchased from Santa

Cruz Biotechnology (Santa Cruz, CA). Mouse anti-STAT3 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-phospho-STAT3 (Tyr705) and anti-phospho-STAT1 (Tyr701) antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit anti-Jak1 and anti-phospho Jak1 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and BioSource (Camarillio, CA), respectively. Alkaline phosphatase-conjugated anti-rabbit or mouse immunoglobulin antibodies (BioSource) were used as secondary antibody and bromochloroindolylphosphate-Nitro blue tetrazolium was the enzyme substrate for Western blotting.

Semi-quantitative RT-PCR

Total cellular RNA was prepared using an RNeasy Mini kit (Qiagen). RT-PCR was performed with the OneStep RT-PCR kit (Qiagen). The quantitative nature of the PCR was validated by the linearity of the determination curve at various concentrations of RNA. The primer sets used to detect SOCS3, SOCS1, CIS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA have been described elsewhere (Sakai et al., 2002; Yokota et al., 2003, 2004a).

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Sensitive and Rapid Detection of Herpes Simplex Virus and Varicella-Zoster Virus DNA by Loop-Mediated Isothermal Amplification

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Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method in which reagents react rapidly and efficiently, with a high specificity, under isothermal conditions. We used a LAMP assay for the detection of herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella-zoster virus (VZV). The virus specificities of primers were confirmed by using 50 HSV-1, 50 HSV-2, and 8 VZV strains. The assay was performed for 45 min at 65°C. The LAMP assay had a 10-fold higher sensitivity than a PCR assay. An analysis of nucleotide sequence variations in the target and primer regions used for the LAMP assay indicated that 3 of 50 HSV-1 strains had single nucleotide polymorphisms. No HSV-2 or VZV strains had nucleotide polymorphisms. Regardless of the sequence variation, there were no differences in sensitivity with the HSV-1-specific LAMP assay. To evaluate the application of the LAMP assay for clinical diagnosis, we tested clinical samples from 40 genital herpes patients and 20 ocular herpes patients. With the LAMP assay, 41 samples with DNA extraction and 26 direct samples without DNA extraction were identified as positive for HSV-1 or HSV-2, although 37 samples with DNA extraction and just one without DNA extraction were positive by a PCR assay. Thus, the LAMP assay was less influenced than the PCR assay by the presence of inhibitory substances in clinical samples. These observations indicate that the LAMP assay is very useful for the diagnosis of HSV-1, HSV-2, and VZV infections.

Herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) and varicella-zoster virus (VZV) are alphaherpesviruses that infect, establish lifelong latency in, and subsequently reactivate from human sensory neuronal ganglia (1, 20). The reactivation of a latent HSV or VZV infection can occur spontaneously or in association with physical or emotional stress and immune suppression. Following reactivation from latent ganglion reservoirs, each of these herpesviruses may cause significant clinical symptoms in the individual and may spread to uninfected persons. Symptomatic VZV reactivation is an infrequent, usually once-in-a-lifetime event in 10% of the population that results in zoster (shingles), while HSV-1 and HSV-2 reactivation occurs frequently and results in numerous symptomatic and asymptomatic recurrences.

HSV-1 and HSV-2 infections and even some VZV infections cause similar clinical symptoms, for example, cutaneous vesicles, keratitis, and acute retinal necrosis (ARN), and the causative agent cannot be distinguished based on the clinical features. However, different clinical courses and prognoses are caused by each virus. HSV-2 genital herpes tends to recur, but HSV-1 genital herpes does not (7). VZV ARN is severer and has a worse prognosis than HSV ARN (4). The identification of the virus is important for the determination of treatment and for an understanding of the clinical progress and prognosis; therefore, an effective laboratory method is urgently needed for the diagnosis of HSV-1, HSV-2, and VZV infections.

At present, molecular methods such as dot blotting, in situ hybridization, and PCR have been developed for pathogen detection. In particular, PCR has been used for numerous recent applications for pathogen detection and pathology research. However, these methods require precision instruments for amplification and DNA extraction, which are the major obstacles to the widespread use of these methods in relatively small-scale clinical laboratories such as those in private clinics. Recently, a new, rapid, and sensitive technique called loop-mediated isothermal amplification (LAMP) was developed (13). LAMP is a nucleic acid amplification method which relies on autocycling strand-displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment. The amplification products are stem-loop DNA structures with several inverted repeats of the target and structures with multiple loops. The LAMP reaction can be conducted under isothermal conditions ranging from 60 to 65°C by using only one type of enzyme and four primers recognizing six distinct regions. The most important merit of this method is that no denaturation of the DNA template is required (11). Moreover, when two primers, termed loop primers, are added, the LAMP reaction time can be shortened (12). With this modification, the LAMP method is able to amplify the target DNA in a shorter time than and with an extremely high specificity compared to the PCR method. Moreover, the LAMP method produces a large amount of amplified product, resulting in easier detection,

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such as detection by visual judgment based on the turbidity or fluorescence of the reaction mixture (10). Recently, several investigators have reported the rapid identification of *Mycobacterium* (6), human herpesvirus 6 (5), human herpesvirus 7 (22), West Nile virus (14), and the severe acute respiratory syndrome coronavirus (3) and have commended the usefulness of the LAMP assay.

For the present study, we used a LAMP assay as the detection system for the diagnosis of HSV-1, HSV-2, and VZV infections. The sensitivity, specificity, and applicability of this method for the direct detection of HSV-1, HSV-2, and VZV from clinical specimens were also evaluated.

MATERIALS AND METHODS

Cells and viruses. An African green monkey kidney cell line (Vero) and human embryonic lung (HEL) fibroblast cells were cultivated in Dulbecco modified Eagle's minimum essential medium (DMEM) supplemented with 10% calf serum. We used 50, 50, and 8 clinically isolated strains of HSV-1, HSV-2, and VZV, respectively. HSV-1 strains were isolated from patients with either genital herpes (n = 20), herpetic keratitis (n = 15), or herpetic conjunctivitis (n = 15). All HSV-2 strains were isolated from patients with genital herpes. The source of the VZV strains is unknown. HSV and VZV strains were isolated on Vero cells and HEL cells, respectively, and stocked at −80°C after a few passages. Each virus was identified and confirmed by a PCR method.

DNA extraction. Virus DNA was extracted from infected cells or clinical samples by proteinase K treatment and phenol-chloroform extraction. After ethanol precipitation, the DNA was suspended in H_2O and stored at −20°C until use.

LAMP method. We used six primers for the LAMP assay, including two outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and two loop primers (LPF and LPB). The location and sequence of each primer are shown in Fig. 1 and Table 1, respectively. FIP consisted of F1 and F2, and BIP consisted of B1 and B2. F3 and B3 were located outside of F2 and B2. Since additional loop primers increase the amplification efficiency, we also synthesized loop primers (LPF and BPF). HSV-1-, HSV-2-, and VZV-specific primers were designed to target the UL1 to UL2, US4, and ORF62 gene regions, respectively. In order to design virus-specific primers, we selected regions of low homology with the nucleotide sequences of the other two viruses.

The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan). A reaction mixture (12.5 μl) containing a 1.6 μM concentration of each inner primer (FIP and BIP), a 0.2 μM concentration of each outer primer (F3 and B3), a 0.8 μM concentration of each loop primer (LPF and BPF), 2× reaction mix (6.25 μl), *Bst* DNA polymerase (0.5 μl), and 1.0 μl of DNA sample was incubated at 65°C for 45 min in a Loopamp real-time turbidmeter (LA-200; Teramecs) and then heated above 80°C for 2 min to terminate the reaction. A portion of each amplified product was analyzed in a 2% agarose gel containing 0.5 μg of ethidium bromide per ml. In addition, the specificity of the LAMP-amplified products was further validated by digestion with a restriction enzyme (PstI for HSV-1 amplicons, HaeIII for HSV-2 amplicons, and AluI for VZV amplicons).

PCR. For an assessment of the sensitivity of the LAMP assay for HSV-1 and HSV-2, PCRs were performed with three primer sets. The first primer set (primer set 1) was B3 and F3, as used in the LAMP assay for HSV-1 and HSV-2 (Table 1). Secondly, we designed a primer set (primer set 2) for HSV-1 and HSV-2 (Table 2). PCR amplification was performed in 15-μl reaction mixtures containing 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, a 500 nM concentration of each primer, and 0.5 unit of *Taq* DNA polymerase. Initial denaturation was done at 95°C for 3 min, followed by 40 cycles of denaturation (30 s at 95°C), annealing (1 min at 55°C and 50°C for primer set 1 and primer set 2, respectively), and extension (45 s at 72°C).

Moreover, in order to check the sensitivity of our PCR assay, we performed PCR amplification by using a commercial primer kit (Maxim Biotech. Inc., Calif.) which has often been used for the diagnosis of HSV in Japanese clinical laboratories (primer set 3). The sequences of these primers were 5′-CATCACCGA CCCGGAGAGGGAC-3′ and 5′-GGGCCAGGCGCTTGTTGGTGTA-3′. PCR amplification with primer set 3 was performed in a 50-μl reaction mixture. Initial denaturation was done at 96°C for 1 min, followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 58°C), and extension (1 min at 72°C). Ten microliters of each reaction mixture was subjected to electrophoresis in a 2% agarose gel containing 0.5 μg of ethidium bromide per ml.

Quantification of HSV DNA. The copy numbers in HSV-1 and HSV-2 DNA samples were analyzed by real-time PCR using a LightCycler (Roche Molecular Biochemicals). For each assay, 2 μl of an extracted DNA sample was added to 18 μl of PCR mixture in each reaction capillary, according to the manufacturer's instructions. The PCR mixture contained 1× FastStart DNA polymerase reaction buffer including deoxynucleoside triphosphates, *Taq* polymerase, and SYBR green I (Roche Molecular Diagnostics), 3 mM $MgCl_2$, and a 0.5 μM concentration of each of the primers. The sequences of the primers for HSV-1 were 5′-TGTGGTCGTCGACGATTGCAGCAT-3′ and 5′-TGGGAGTGACCCGC GTGGTCGA-3′, and those for HSV-2 were 5′-CTACGACGCGTACCGGTC CGATG-3′ and 5′-GTGGTGCACGAACAGCGTGGTGA-3′. After denaturation for 10 min at 95°C, 40 cycles of amplification at 95°C for 0 s, 55°C for 10 s, and 72°C for 20 s were carried out. The amount of amplified product for the targeted nucleic acid was monitored by SYBR green I staining during each cycle of the PCR. Prediluted standard DNA templates comprising 10^3 to 10^7 copies of a quantified PCR product were applied to the real-time PCR analysis to make a standard curve. The copy numbers of HSV-1 and HSV-2 DNA were then determined by using the standard curve.

DNA sequencing of virus strains. The nucleotide sequences of the LAMP target and primer regions in the HSV-1, HSV-2, and VZV strains were determined by a PCR-directed sequencing method. Briefly, double-stranded DNA templates for sequencing reactions were prepared by PCRs using the primer sets shown in Table 2 as described previously (18). PCR products from the HSV-1, HSV-2, and VZV strains were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and used as templates for DNA sequencing reactions. Nucleotide sequencing was performed by use of an autosequencer (ABI PRISM 3100 genetic analyzer) and a commercial kit (BigDye; Applied Biosystems, Foster City, Calif.).

Clinical samples. We evaluated samples for 40 cases of genital herpes and 20 cases of ocular herpes. The genital samples were vaginal smears from patients suspected of having genital herpes. Ocular herpes samples were obtained from 5 patients with herpetic dendritic keratitis and 15 patients with uveitis and suspected ARN. Keratitis samples were corneal smears and uveitis samples were from aqueous (n = 6) and vitreous (n = 9) fluid. The smear samples were scraped with a cotton swab and placed in 0.5 ml of DMEM. Aqueous samples were obtained by anterior chamber paracentesis. Vitreous samples were obtained at the time of surgery. All samples were stored at −80°C until use. LAMP, PCR, and virus isolation were performed for all clinical samples. The sequences of the PCR primers are summarized in Table 2.

RESULTS

Specificity of LAMP assay. A successful LAMP reaction with virus-specific primers demonstrated many bands of different sizes upon agarose gel electrophoresis (Fig. 2). When the sample tube did not contain target DNA, no amplification was seen. To confirm that the products were amplified from the target region, we digested the products with a restriction enzyme, i.e., PstI for HSV-1, HaeIII for HSV-2, and AluI for VZV. The sizes of the fragments were in good agreement with those predicted theoretically from the expected DNA structures and showed that the reaction was specific.

Sequence variation of LAMP primer and target regions. After PCR-directed sequencing of the LAMP target and primer regions, 3 HSV-1 strains, comprising 1 strain causing keratitis (KH15), 1 strain causing conjunctivitis (conj10), and 1 strain causing genitalitis (G1-4), of the 50 total strains had single nucleotide polymorphisms (Fig. 1). The polymorphism sites of the three strains were unique. The variations were in the F3 region of the G1-4 strain, the F1 region of the conj10 strain, and the B1 region of the KH15 strain. No HSV-2 or VZV strains had nucleotide polymorphisms. Thus, we speculated that our LAMP target and primer regions for HSV-1, HSV-2, and VZV had low levels of nucleotide sequence variation.

Sensitivity of LAMP assay for HSV-1 and HSV-2. The sensitivity of the LAMP assay for HSV-1 and HSV-2 was determined by testing serial 10-fold dilutions of samples for which

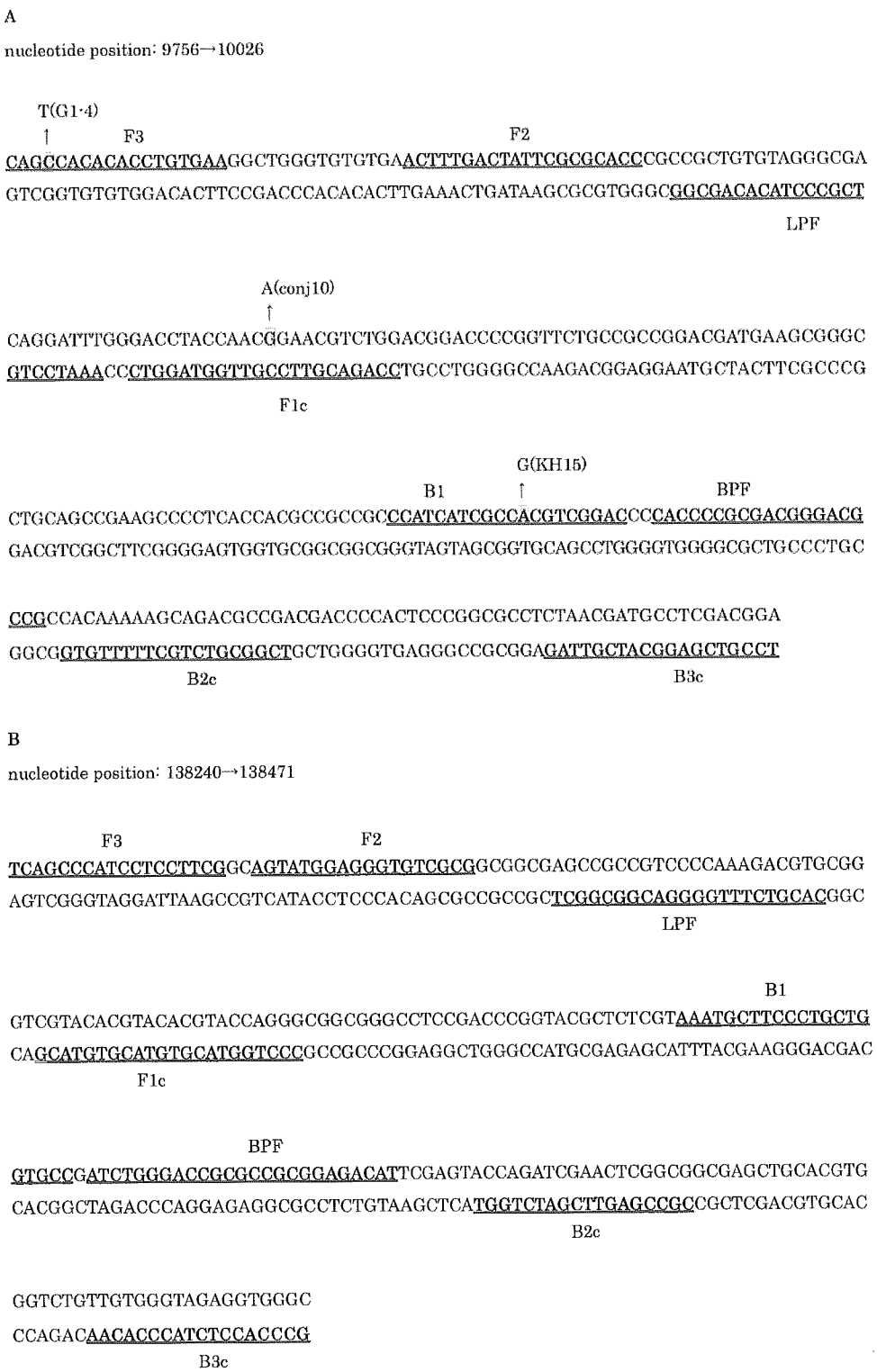

FIG. 1. Location and sequence of LAMP target and primer regions for HSV-1 (A), HSV-2 (B), and VZV (C). The single nucleotide polymorphisms of three HSV-1 strains (the G1-4, conj10, and KH15 strains) are shaded. Arrows indicate the nucleotide bases of strains with polymorphism.

the copy numbers of virus DNA had previously been quantified by real-time PCR and comparing the results with those of PCR. The analyzed strains showed 100% nucleotide sequence homology in the LAMP primer region. In order to check the sensitivity of PCR accurately, we carried out a PCR assay using three primer sets. Primer set 1 consisted of B3 and F3 from the HSV-1 and HSV-2 LAMP assays, with which regions of low nucleotide sequence variation were proven in this study. The

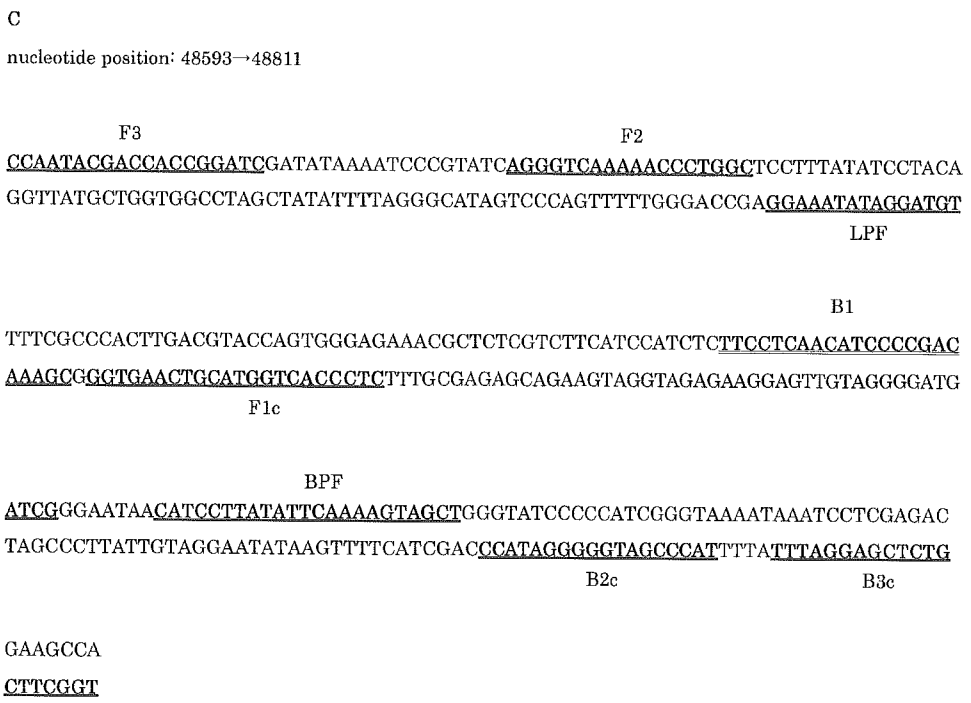

FIG. 1—*Continued.*

sensitivity of this primer set was much lower than those of primer set 2 and primer set 3 (data not shown). The PCR assay using primer set 2 or primer set 3 had a detection limit of 100 copies/tube. Therefore, we used primer set 2 for this study.

The HSV-1 and HSV-2 LAMP assay could detect as few as 10 copies/tube, which was a 10-fold higher sensitivity than that of the PCR assays with primer set 2 (Table 2) and primer set 3. We studied the influence of nucleotide polymorphisms in the primer region on the sensitivity of the LAMP assay by using three HSV-1 strains. The detection limit for all three strains was 10 copies/tube, and there were no differences in the sensitivity of the LAMP assay for HSV-1, regardless of the presence of single nucleotide variations (Fig. 3).

TABLE 1. Primers used for LAMP

Virus	Primer	Sequence (5′-3′)
HSV-1	F3	CAGCCACACACCTGTGAA
	B3	TCCGTCGAGGCATCGTTAG
	FIP(F1-F2)	CCAGACGTTCCGTTGGTAGGTC-ACTTTGACTATTCGCGCACC
	BIP(B1-B2)	CCATCATCGCCACGTCGGAC-TCGGCGTCTGCTTTTTGTG
	LPF	AAATCCTGTCGCCCTACACAGCGG
	LPB	CACCCCGCGACGGGACGCCG
HSV-2	F3	TCAGCCCATCCTCCTTCG
	B3	GCCCACCTCTACCCACAA
	FIP(F1-F2)	CCCTGGTACGTGACGTGTACG-AGTATGGAGGGTGTCGCG
	BIP(B1-B2)	AAATGCTTCCCTGCTGGTGCC-CGCCGAGTTCGATCTGGT
	LPF	CACGTCTTTGGGGACGGCGGCT
	LPB	ATCTGGGACCGCGCCGCGGAGACAT
VZV	F3	CCAATACGACCACCGGATC
	B3	TGGCTTCGTCTCGAGGATTT
	FIP(F1-F2)	CTCCCACTGGTACGTCAAGTGG-AGGGTCAAAAACCCTGGC
	BIP(B1-B2)	TTCCTCAACATCCCCGACATCG-TACCCGATGGGGGATACC
	LPF	CGAAATGTAGGATATAAAGG
	BPF	CATCCTTATATTCAAAAGTAGCT

Evaluation and comparison of LAMP and PCR for detection of DNA in clinical samples. Previous reports have described clinical samples as containing some PCR inhibitors (8, 9, 15, 21); therefore, DNA preparation is required to obtain sensitive results for PCR assays. In order to clarify whether a DNA preparation procedure was necessary to obtain sensitive results with LAMP, we tested 40 genital and 20 ocular samples, with and without a DNA extraction procedure, by both the LAMP and PCR assays. For the assay of genital herpes samples, 30 samples subjected to DNA extraction were detected as HSV-1 or HSV-2 by both PCR and LAMP, indicating that the methods possessed equal sensitivities (Table 3). Of these 30 samples, HSV was isolated from 24 samples. Using direct sample fluid, we detected HSV-1 or HSV-2 DNA in 21 samples by the LAMP assay, but in just 1 sample by PCR, showing that the LAMP assay was less influenced by inhibitors than was PCR. For the assay of ocular herpes samples, virus DNA was detected by LAMP in 11 of 20 samples subjected to DNA extraction. However, the HSV genome was not detected in four LAMP-positive uveitis samples by PCR. With direct sample fluid, five cases were diagnosed as HSV infections by the LAMP assay, but HSV was not detected in the ocular samples by PCR (Table 3).

DISCUSSION

We used a novel nucleic acid amplification method, LAMP, for the detection of HSV-1, HSV-2, and VZV. With the method used, viruses could be identified within 45 min of DNA

TABLE 2. Primers used for PCR

Virus	Primer	Size of PCR product (bp)	Positions	Sequence (5′-3′)
HSV-1	HSV1-seqF	511	9658–9680	AGTTACCCCGCGTTTCCTGCCAA
	HSV1-seqR		10168–10145	GTTTGTTAAATCCATGGGAGGGTC
HSV-2	HSV2-seqF	637	138010–138034	GTGGCTCAATATTGTTATGCCTATC
	HSV2-seqR		138646–138626	TCTGTGGGGGCAGACAGCGCT
VZV	VZV-seqF	556	48461–48485	GACGTTTTCCTCCAACTGTAAAGGT
	VZV-seqR		49016–48993	ATTCCGGGATATTACGCTAGTGGA

extraction. The LAMP operation is quite simple; it starts with the mixing of buffer, primers, DNA lysates, and DNA polymerase in a tube, and then the mixture is incubated at 65°C for a certain period. There is no need for a thermal cycler because there is no heat denaturation step of the template DNAs with this method. The only equipment needed for the LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature of 65°C. Moreover, the sensitivity of the LAMP assay for HSV-1 and HSV-2 was 10 times higher than that of PCR assays.

The nucleotide sequences of HSV-1 and HSV-2 have high homology, and regions of nucleotide sequence specificity between HSV-1 and HSV-2 are limited. We found sequence specificity for HSV-1 and HSV-2 in the UL1 to UL2 genes and the US4 gene, respectively; therefore, we designed primers to target these regions. The sequences of the HSV-1-specific forward primers (F3, FIP, and LPF) have high homology to HSV-2, but the reverse primers (B3, BIP, and BPF) are specific for HSV-1. All HSV-2-specific LAMP primers had a low homology with HSV-1. The virus specificities of these primers were confirmed in our study.

An evaluation of three strains with single nucleotide sequence variations showed that there was no difference in the sensitivity of the LAMP assay for these HSV-1 strains. Sakaoka et al. reported that the nucleotide diversity of HSV-1 was 0.0037 (3 or 4 nucleotides per 1,000 bases) between any two strains (16). The nucleotide diversity in the LAMP target and primer regions of 271 bp of 50 HSV-1 strains was 0.00044 (4 or 5 nucleotides per 10,000 bases). Thus, the LAMP target and primer regions of HSV-1 are regarded as having adequately low degrees of variation. Compared to HSV-1, HSV-2 and VZV have little nucleotide diversity among strains (2, 17, 19). Our sequencing study indicated that the targeted regions of HSV-2 and VZV have no nucleotide sequence variation and are also adequate for the LAMP assay.

In an evaluation of clinical samples, 30 of 40 patients suffering from genitalitis were identified as having HSV-1 or HSV-2 infection by a LAMP assay with samples subjected to DNA extraction. We could not diagnose whether the other 10 patients suffered from HSV infection or other diseases. In the case of keratitis, the diagnosis of herpes keratitis is relatively easy on the basis of its typical symptom, dendritic keratitis, so the frequency of HSV infection was high (four of five cases). However, the frequency of HSV detection in uveitis samples was low because the frequency of ARN during uveitis is low.

We paid attention to and evaluated the two methods by

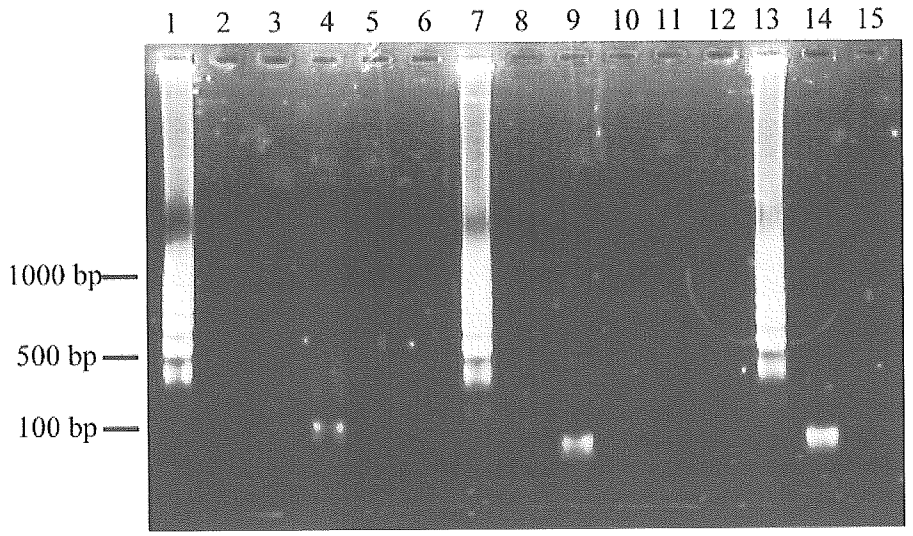

FIG. 2. Electrophoretic analysis of LAMP-amplified products. Lanes 1, 2, and 3, LAMP carried out with HSV-1 primers and genomic DNAs from HSV-1, HSV-2, and VZV, respectively; lane 4, LAMP product from lane 1 after digestion with PstI; lanes 5, 10, and 15, LAMP carried out in the absence of template DNA with the HSV-1, HSV-2, and VZV primers, respectively; lanes 6, 7, and 8, LAMP carried out with HSV-2 primers in the presence of genomic DNAs from HSV-1, HSV-2, and VZV, respectively; lane 9, LAMP product from lane 7 after digestion with HaeIII; lanes 11, 12, and 13, LAMP carried out with VZV primers in the presence of genomic DNAs from HSV-1, HSV-2, and VZV, respectively; lane 14, LAMP product from lane 13 after digestion with AluI. The positions of size markers are shown on the left.

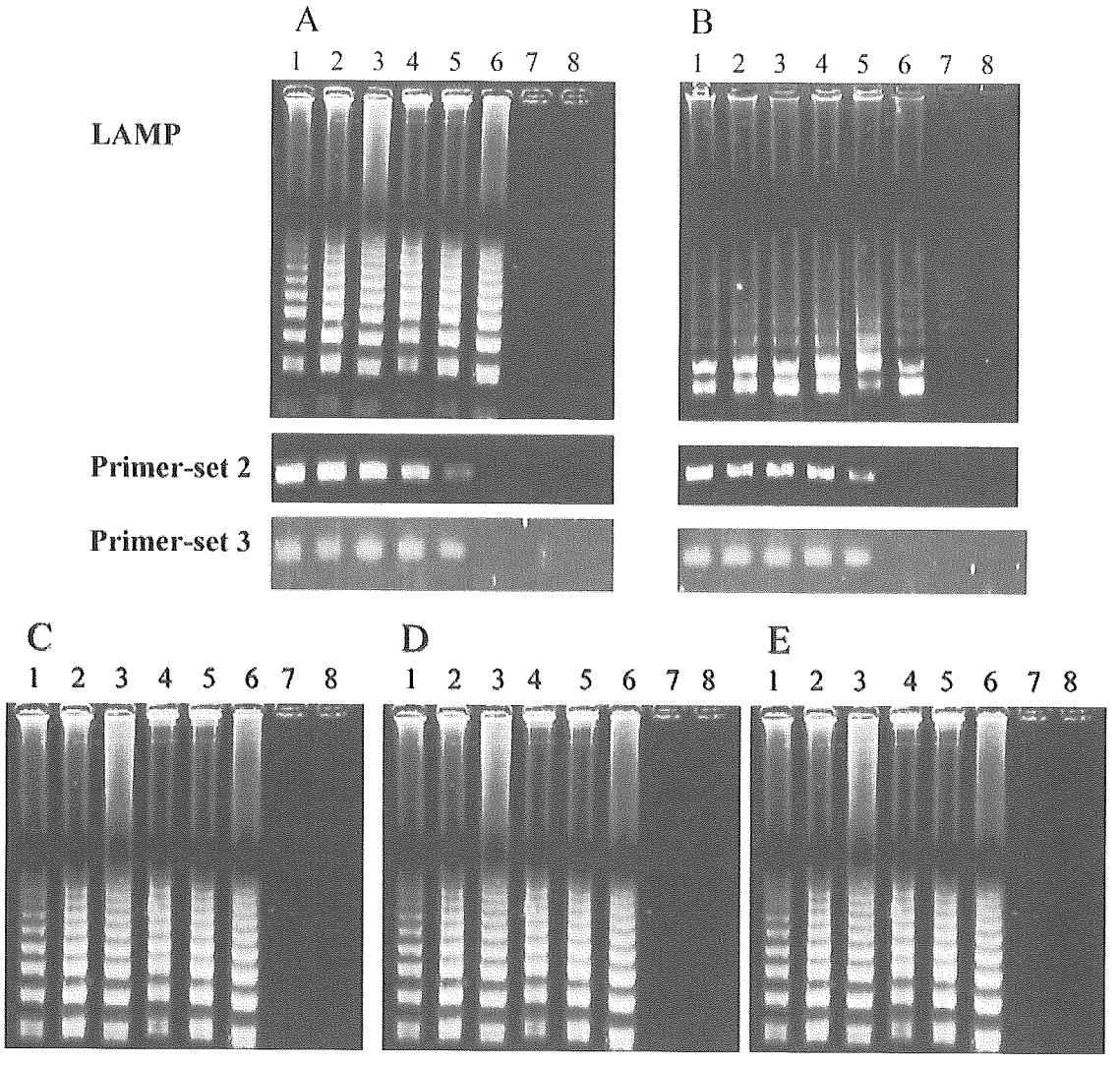

FIG. 3. Comparative sensitivities of LAMP and PCR for the detection of HSV-1 (A) and HSV-2 (B). Amplification by LAMP shows a ladder-like pattern, whereas that by primer set 2 and primer set 3 shows single bands for the amplification products obtained by using the primer set shown in Table 2 and that from a commercial PCR kit, respectively. (C to E) Comparative sensitivities to single nucleotide polymorphisms of three HSV-1 strains. The electrophoretic profiles of the G1-4 strain (C), the conj10 strain (D), and the KH15 strain (E) are shown. Lanes: 1, 10^6 copies/tube; 2, 10^5 copies/tube; 3, 10^4 copies/tube; 4, 10^3 copies/tube; 5, 10^2 copies/tube; 6, 10^1 copies/tube; 7, 10^0 copies/tube; 8, negative control without target DNA.

using clinical samples. We first evaluated the sensitivity of LAMP compared to that of PCR, using samples subjected to DNA extraction. Both LAMP and PCR showed the same sensitivity for identifying genital herpes and herpetic keratitis. However, three of seven LAMP-positive uveitis samples were negative in the PCR assay. This indicated that the sensitivity of LAMP is high enough to detect low DNA copy numbers and is therefore useful for small-volume samples such as those of vitreous and aqueous humor. Next, we evaluated the necessity of DNA extraction. Biological fluids, e.g., aqueous and vitreous humor, contain substances that inhibit PCR (8, 9, 15, 21). Therefore, samples for PCR assays require DNA extraction. Using direct sample fluid (in DMEM) with genital smear suspensions, we identified HSV-1 or HSV-2 in 21 samples by LAMP and in just 1 sample by PCR. Using direct ocular sample fluid, we found that 5 of the 11 samples that were

TABLE 3. Comparison of LAMP assay and PCR assay by using clinical samples

Condition (no. of samples)	Virus	No. of DNA samples with positive result[a]		No. of direct samples with positive result[b]		No. of isolated samples
		LAMP	PCR	LAMP	PCR	
Genitalitis (40)	HSV-1	17	17	12	1	15
	HSV-2	13	13	9	0	9
Keratitis (5)	HSV-1	4	4	2	0	2
	HSV-2	0	0	0	0	0
Uveitis (15)	HSV-1	2	1	1	0	0
	HSV-2	5	2	2	0	0

[a] Samples subjected to DNA extraction.
[b] Direct sample fluid without DNA extraction.

positive after DNA extraction were also positive in the LAMP assay, but none were positive in the PCR assay. The inhibitory substances reduced the sensitivity of PCR, resulting in a PCR sensitivity which was lower than that required for virus isolation. These results indicate that the LAMP assay is more tolerant of substances contained in clinical specimens than is PCR.

In conclusion, the LAMP assay is a simple, rapid, and accurate nucleoside amplification method that is useful for the clinical diagnosis of HSV and VZV infection. Compared to PCR, the LAMP assay possesses advantages in its sensitivity and tolerance of inhibitory substances. These results suggest that the LAMP assay could be introduced into clinical laboratories and even private clinics.

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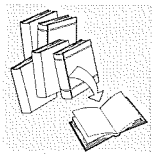
REVIEW



Neuropathogenesis in cytomegalovirus infection: Indication of the mechanisms using mouse models

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SUMMARY

Cytomegalovirus (CMV) is the most frequent infectious cause of developmental brain disorders and also causes brain damage in immunocompromised individuals. Although the brain is one of the main targets of CMV infection, little is known about the neuropathogenesis of the brain disorders caused by CMV in humans because of the limitations in studying human subjects. Murine CMV (MCMV) is similar to human CMV (HCMV) in terms of genome structure, pattern of gene expressions, cell tropism and infectious dynamics. In mouse models, it has been shown that neural stem/progenitor cells are the most susceptible to CMV infection in developing brains. During brain development, lytic infection tends to occur in immature glial cells, presumably causing structural disorders of the brain. In the prolonged phase of infection, CMV preferentially infects neuronal cells. Infection of neurons may tend to become persistent by evasion of immune reactions, anti-apoptotic effects and neuron-specific activation of the e1-promoter, presumably causing functional neuronal disorders. It has also been shown that CMV infection in developing brains may become latent in neural immature cells. Brain disorders may occur long after infection by reactivation of the latent infection. Copyright © 2005 John Wiley & Sons, Ltd.

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INTRODUCTION

Cytomegalovirus (CMV), a member of the herpes virus group, is the most significant infectious cause of congenital anomalies in the central nervous system (CNS) caused by intrauterine infection in humans, with an average incidence of approximately 1.0% live births [1–4]. It is estimated that approximately 5%–10% of infected infants have generalised cytomegalic inclusion disease at birth, with symptoms such as microcephaly, periventricular calcification [2], and eye abnormalities such as micropthalmia and optic nerve atrophy [5]. Of the other infected infants nearly 10% have subclinical congenital infection but will subsequently develop brain disorders including mental retardation, sensorineural hearing loss, visual defects, seizures and epilepsy [6,7]. In adults, infection with human CMV (HCMV) is asymptomatic in immunocompetent hosts, but the virus causes severe or fatal disease in immunocompromised patients [8,9]. CMV has become the most frequent opportunistic cerebral infection in acquired immunodeficiency syndrome (AIDS), in which it results in CMV encephalitis/encephalopathy such as ventriculo-encephalitis [10–12]. The brain is a major target in congenital CMV infection and immunocompromised patients [9].

CMV infection demonstrates a strict host cell type and species specificity [13–15]. As a consequence of the difficulties associated with studies of the pathogenesis of HCMV infection in humans, murine CMV (MCMV) was chosen as a model for HCMV infection. The major reason for using MCMV as a model is the matching biological characteristics of these virus infections in their natural setting. Both MCMV and HCMV cause

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Abbreviations used
CMV, cytomegalovirus; CNS, central nervous system; CP, cortical plate; β-gal, β-galactosidase; GFAP, glial fibrillary acidic protein; X-Gal, 5-bromo-3-chloro-indolyl-β-galactoside; HCMV, human cytomegalovirus; IE, immediate-early; MCMV, murine cytomegalovirus; NMDA, N-methyl-D-aspartate; NSE, neuron-specific enolase; NSPC, neural stem/progenitor cells; SVZ, subventricular zone; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VZ, ventricular zone.

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severe infections in the immunocompromised or immunologically immature host, resulting in similar clinical syndromes [16]. Both viruses have large genomes consisting of about 230 kilobase pairs (kbp) retaining colinear genome organisation: HCMV (AD169) was 229354 bp [17], whereas MCMV (smith strain) was 230278 bp [16]. Both the HCMV and MCMV genomes contain 165–170 predicted open reading frames (ORFs) [16,18]. Despite significant differences in the overall arrangement of the genomes of MCMV and HCMV, the genomes are very similar at the genetic and nucleotide composition level [16].

Model systems have been developed for brain abnormalities induced by infection of mouse embryos with MCMV [19–21]. Based on these models, this review focuses on the effects of MCMV infection on embryogenesis and brain development, and also on neural proliferation and differentiation. These studies may provide basic mechanisms for neuropathogenesis of brain disorders induced by CMV in the human.

SUSCEPTIBILITY OF DEVELOPING MOUSE BRAINS TO MCMV

In the early embryonic stage in the mouse, no sign of infection was detected after injection of MCMV into blastocysts and returning them to the uterus [22,23]. Early embryos were also not susceptible to Moloney murine leukemia virus [24] and relatively resistant to West Nile virus (WNV) [25]. It is possible that early embryos may be protected from CMV infection for preservation of the species.

In congenital CMV infection in humans, the brain is the preferential site of infection resulting in sequelae such as microcephaly and microphthalmia as morphological abnormalities, and sequelae such as mental disorder or epilepsy as functional disorders [9,26]. However, little is known about the timing of brain susceptibility to CMV and the susceptible cells in the brain. As an experimental system, the susceptibility of the developing brain to MCMV has been shown in mice by infection of MCMV into embryos through the placenta or by intraperitoneal infection of the virus into the fetus.

The placenta is regarded as a site of congenital CMV infection and infection of the placenta is thought to be a necessary step for transmission of CMV infection into fetuses [27,28]. It has been reported that transplacental transmission with MCMV does not occur in the mouse [29] presumably because of the three trophoblastic layers of the mouse placenta, which is different from the placentas of human and guinea pigs. In order to simulate transplacental infection to fetuses, mouse placentas at embryonic day 12.5 of gestation (E12.5) were injected directly with MCMV, then the embryos were allowed to develop until E18.5 [21]. The frequency of infection in the brains was prominent in the embryos with placental infection, which was the same as that in the liver and higher than that in the lungs (Figure 1A). In the embryonic brains, the cerebral ventricular zone is the most susceptible site for MCMV infection (Figure 1B). Developmental retardation with microcephaly was sometimes observed in offspring exposed to the placental infection (Figure 1C). Although the ventricular wall has been thought to be a target of congenital CMV infection in humans [2,26] and in animal models [20], direct evidence was provided that fetal brains can be infected by placental infection in mice.

It is known that the susceptibility of mice to MCMV infection *in vivo* diminishes with age [30]. When fetal and neonatal mice were injected intraperitoneally with MCMV during the course of development, fetal and perinatal mice were the most susceptible to MCMV infection [31]. Therefore, the earlier in the developmental stage of the mouse, the more susceptible is the brain to MCMV infection in this experimental system. The brain may acquire some factors for resistance to CMV infection during development, which diminishes susceptibility.

NEURAL STEM/PROGENITOR CELLS AS TARGETS FOR SUSCEPTIBILITY

Resistance to susceptibility to CMV has been thought to be due to development of host defense mechanisms such as those mediated by natural killer (NK) cells [32] and macrophages [33] including microglia. It is rather difficult to standardise experimental conditions for *in vivo* analysis of brain susceptibility to virus infection. However, the susceptibility of cells to CMV infection *in vivo* is markedly different from that *in vitro* [8].

Susceptibility in brain slice cultures

Brain slice cultures provide a useful experimental system because they preserve the three-dimensional

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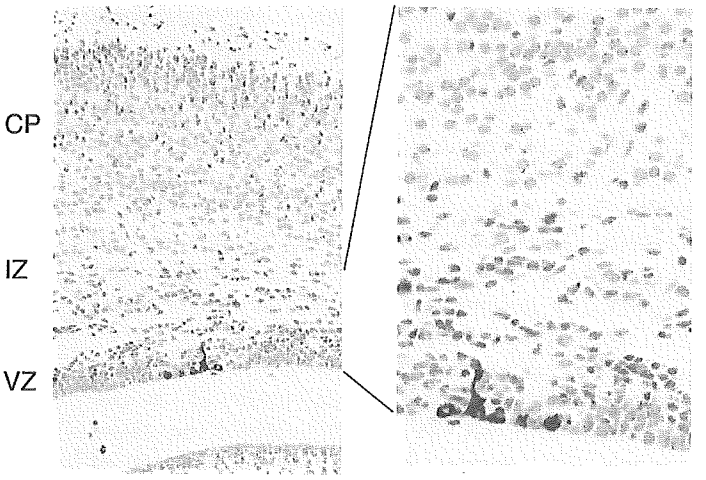
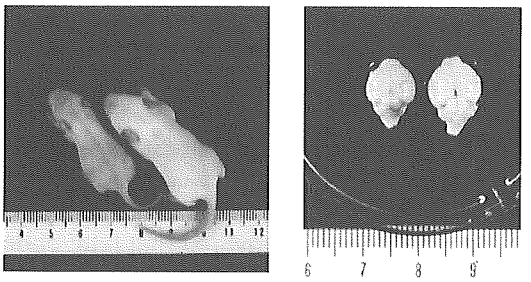
A Placentas and fetus after placental infection with MCMV

	MCMV		MEM	
	PCR (%)	IHC (%)	PCR (%)	IHC (%)
Placenta	22 / 36 (61.1)	16 / 26 (61.5)	1 / 13 (7.7)	0 / 6 (0)
Fetus	13 / 36 (36.1)	8 / 26 (30.8)	0 / 10 (0)	0 / 10 (0)
Brain	10 / 36 (27.8)	7 / 26 (26.9)	0 / 10 (0)	0 / 10 (0)
Lung	7 / 36 (19.4)	4 / 26 (15.4)	0 / 3 (0)	0 / 10 (0)
Liver	10 / 36 (27.8)	7 / 26 (26.9)	0 / 10 (0)	0 / 10 (0)
Survival rate	36 / 62 (58.1)		29 / 48 (60.4)	

B MCMV-infected cells in fetal brain



C Postnatal mice and brains



Growth retardation Microcephaly

Figure 1. Mouse fetuses and brains after placental infection with MCMV. (A) Numbers of infected placentas and fetuses. At day 12.5 of gestation (E12.5), MCMV (1×10^3 PFU) was injected into placentas of the right uterine horn and MEM was injected into placentas of the left horn. The placenta and fetuses were examined at E18.5 by PCR for viral genome and immunohistochemical staining (IHC) for viral antigen. (B) Immunostaining of MCMV-infected cells in the fetal brain at E18.5. CP (cortical plate), IZ (intermediate zone), VZ (ventricular zone). (C) Growth retardation and microcephaly in 7-day-old offspring mice after placental infection with MCMV at E12.2

architecture and the local environment of the brain cells [34], including neurons, glial cells and other immune cells, to a greater extent than dissociated cell cultures. In addition, they permit experimental manipulations and observations to be performed [35]. The effects of MCMV infection on developing mouse brains was investigated in terms of susceptible cells and age-related resistance to MCMV in brain slice cultures [36,37]. Brain slices from BALB/c mice at different developmental stages were infected *in vitro* with recombinant MCMV (RM461) in which the lacZ gene was inserted into a late gene (provided by Dr E.S. Mocarski, Stanford University) [38]. The infected cells can be detected by X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside) and β-gal (β-galactosidase) expression and was visualised as blue spots on brain slices. The subventricular zone (SVZ) and cortical marginal region were the sites most susceptible to MCMV infection, and the susceptibility declined with the development of the brain as seen *in vivo* (Figure 2A). It has been reported that resistance to MCMV infection is due to the development of host defense mechanisms such as NK cells [32]. The NK protection against MCMV infection is in part related to a defined resistance gene locus, Cmv-1, mapping to chromosome 6 in the NK complex region [39,40]. It was reported that C57BL/6 mice, which have the Cmv-1 resistant allele, are less susceptible to MCMV infection than BALB/c, which do not have the allele. However, the susceptibility to MCMV infection of brain slice cultures from BALB/c mice was not different from that of slices from C57BL/6 mice [37]. Furthermore, the susceptibility of the developing brains to MCMV in immunocompromised SCID mice was not different from that of BALB/c mice although the mortality rate of SCID mice was substantially greater than BALB/c mice [41]. This Beige-SCID mouse is deficient in both T and B cell functions and has reduced natural killer

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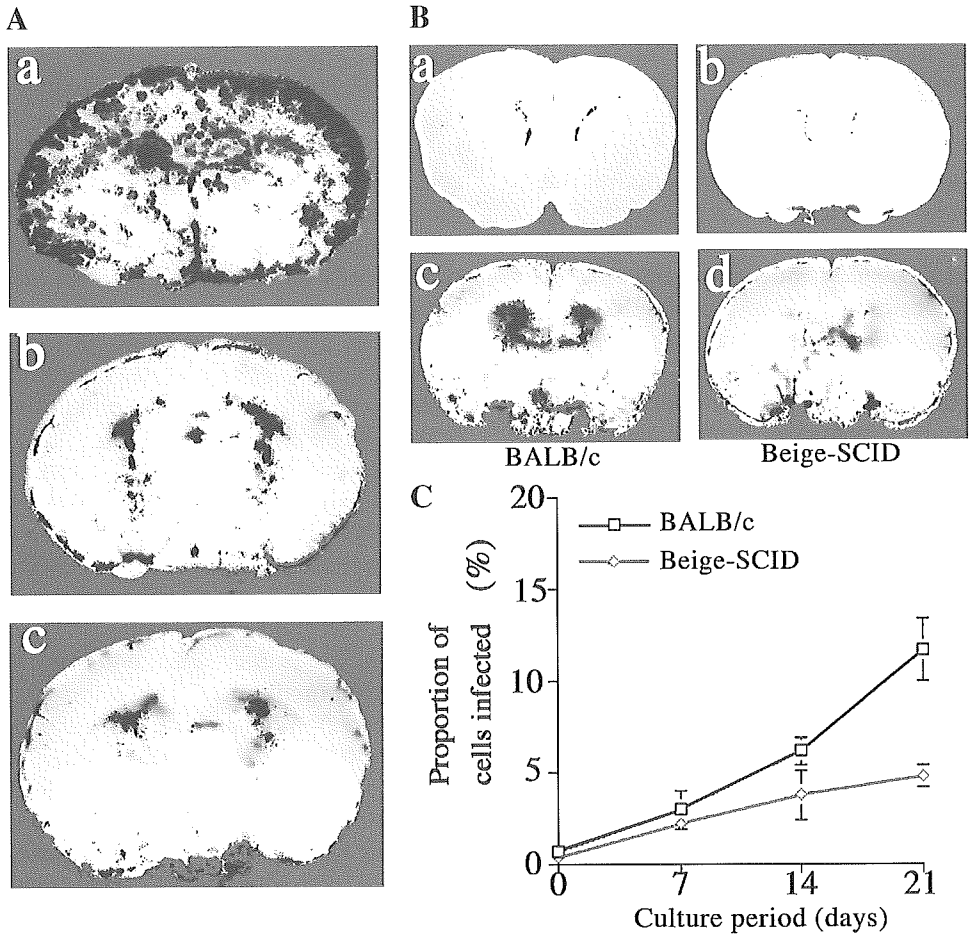

Figure 2. (A) Age-dependent changes of β-Gal expression in the brain slice cultures from mice of different ages after MCMV infection. The brain slice cultures from 7-day-old (a), 14-day-old (b) and 21-day-old (c) BALB/c mice were infected with recombinant MCMV (RM461) (5×10^6 PFU/ml) *in vitro*, and cultured for 5 days, fixed and stained with X-Gal. (B) Increase of infected cells by prolonged culturing prior to MCMV infection. Brain slices from 21-day-old BALB/c mice (a, c) and Beige-SCID mice (b, d) were cultured for 0 day (a, b) and 21 days (c, d) prior to infection with mutant MCMV (RM461). After infection the slices were incubated for 5 days. The number of X-Gal-positive cells increased when the culture period was longer prior to infection. (C) Comparison of the susceptibility of brain slices from BALB/c with that of slices from Beige-SCID mice. Quantitative analysis of proportion of cells infected in brain slices from BALB/c and Beige-SCID mice 5 days after infection by estimating the X-Gal-stained area per whole brain slice area by using Photoshop image software

cell activity [42]. The cells susceptible to MCMV in the ventricular zone proliferated when the brain slices were cultured for a prolonged time. Unexpectedly the amount of proliferation of the susceptible cells was lower in the Beige-SCID mice than in the BALB/c mice [43] (Figure 2B, C). It is possible that immunologic defense mechanisms to MCMV infection work poorly in the developing brain, independent of whole systemic immune system.

Immunostaining showed that virus-susceptible cells in the subventricular zone (SVZ) and cortical marginal regions were positive for nestin, Musashi and glial fibrillary acidic protein (GFAP), and that most of the infected cells were positive for the proliferating cell nuclear antigen (PCNA) and labeled with bromodeoxyuridine (BrdU) [37]. Thus, the SVZ is the most susceptible site in the brain to MCMV infection and the susceptible cells in the regions are immature neural cells, including neural stem/progenitor cells. Most of the cells in the SVZ consist of undifferentiated neuroepithelial cells, which include neural stem/progenitor cells [44–46]. These cells express neural stem cell markers such as nestin and Musashi [47,48] and also

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GFAP [45]. However, these neural stem cell markers were reported to be expressed also in neural progenitor cells and immature glial cells [46]. Therefore, it is possible that cells susceptible to MCMV in the SVZ are immature glial cells and neural progenitor cells, including neural stem cells. It was also reported that in the experiments with rhesus macaques, the spectrum of cortical anomalies and the distribution of infected cells in the fetal brain tissue indicated that immature neuronal cells were preferential targets for rhesus CMV (RhCMV) infection [49]. Furthermore, the targets for human herpesvirus 6 (HHV-6) were reported to be human glial precursor cell culture [50] and targets for coxsackievirus B3 are immature neural cells in the developing brains of BALB/c mice [51].

Susceptibility of neurospheres to MCMV

Since immature neural progenitor cells are important factors in the determination of susceptibility of the developing brain to MCMV, the effect of MCMV on the proliferation and differentiation of neural stem cell cultures was analysed according to the method of Reynolds and Weiss [52,53]. In the ventricular zone, multipotential neural stem cells have been identified *in vivo* and *in vitro*, and were shown to be capable of proliferation, self-renewal and to have the potential to differentiate to neuronal or glial precursors [47,54]. The isolated primary brain cells are able to proliferate from single cells to form multipotential spherical cell masses called neurospheres in the presence of epidermal growth factor (EGF). Later neurospheres were proved to be composed of not only neural stem cells but also neural progenitor cells [46,55], therefore, cells in neurospheres are better named neural stem/progenitor cells (NSPC).

When neural stem/progenitor cells were infected with MCMV as single cells and cultured, the shape of the neurospheres was distorted, viral antigens were expressed in the cells of neurospheres (Figure 3A), and then infectious viruses were released from the neurospheres as detected by plaque assay [56]. It was found that neural stem/progenitor cells are permissive for MCMV infection, although MCMV replication was slower than in mouse embryonic fibroblasts (Figure 3, left upper). MCMV infection inhibited the growth (Figure 3B, right upper) and DNA replication of the neural stem/progenitor cells. A clonogenic assay revealed that MCMV infection suppressed the generation of colonies from single stem cells (Figure 3, left lower). Because a single cell having the potential to form a neurosphere is thought to be a neural stem cell but not a neural progenitor cell, the results described above may apply only to neural stem cells. It is worth noticing that neural stem cells are susceptible to MCMV infection unlike embryonic stem (ES) cells (in preparation for publication). It has been reported that neural stem cells are different from pluripotent ES cells in terms of genetics and functions [57,58]. These differences between neural stem cells and ES cells remain to be clarified.

In the undifferentiated condition, neurospheres express only nestin but not GFAP and neurofilaments. Neural stem/progenitor cells are induced to differentiate by removing growth factor EGF and adding 2% horse serum, and the differentiated cells are attached on the bottom. Then the expression of nestin is decreased, whereas the expression of neurofilament and GFAP is induced (Figure 3B, right lower). When neural stem/progenitor cells were induced to differentiate after MCMV infection, nestin expression was retained, whereas the expression of neurofilament was more strongly inhibited than that of GFAP in these cells [56]. It is noteworthy that neural stem/progenitor cells tend to retain their undifferentiated condition following MCMV infection. After transplantation into the developing brain, MCMV infection inhibited the migration and neuronal differentiation of the neural stem/progenitor cells [56]. It is possible that CMV infection supports the permissiveness of neural stem cells, inhibits the growth of the neural stem/progenitor cells, and suppresses the neuronal differentiation in addition to disturbing neuronal cell migration. These potential effects may offer an explanation for the developmental disorders of brains associated with congenital CMV infection in humans such as microcephaly.

CELL TROPISM OF VIRAL GENE EXPRESSION IN GLIAL AND NEURONAL CELLS

During brain development, neural stem cells commit to induce neural progenitor cells in the ventricular zone (VZ), and migrate to the cortical plate with differentiation to glial or neuronal cells, then forming a cortical layer [59]. It is important to know how CMV infection affects

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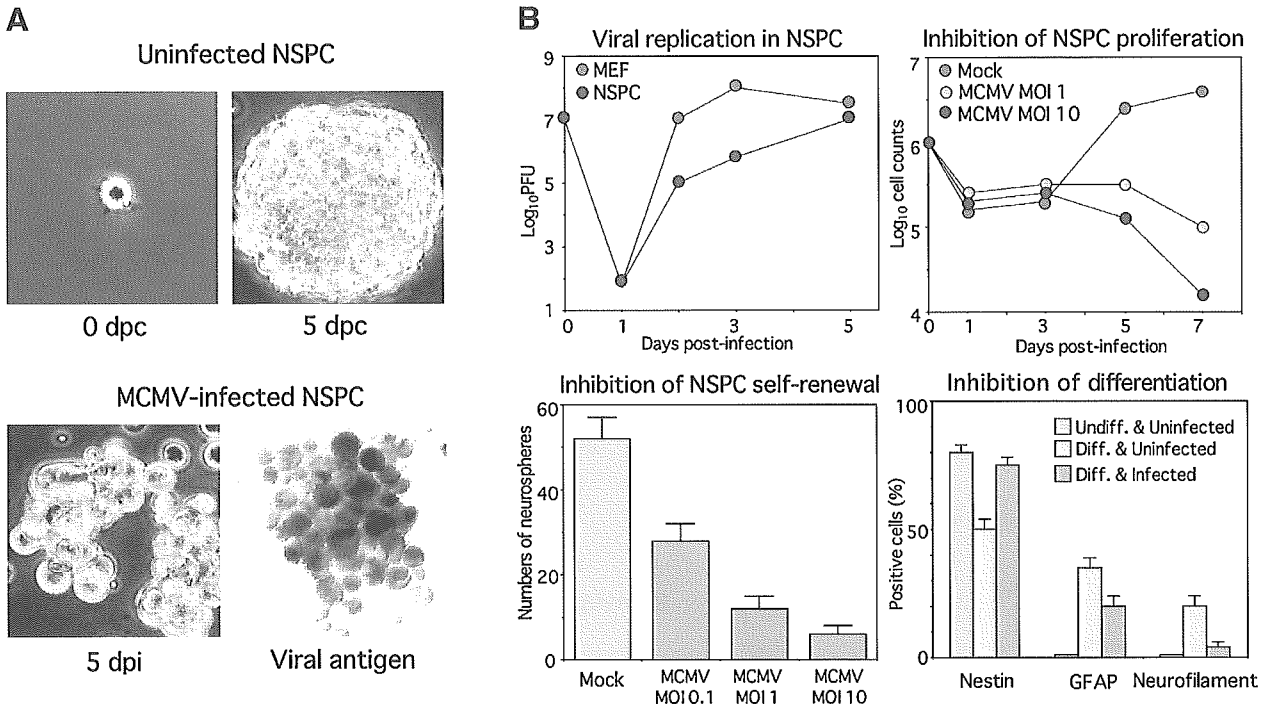

Figure 3. Effect of MCMV infection on cultured neural stem/progenitor cells (NSPC) (neurospheres). (A) Effect of MCMV infection on shape and expression of the viral antigen of NSPC. A NSPC from fetal brain (E15.5) formed a neurosphere 5 days after culture (upper). Cells were infected with MCMV as single cells. Shape of neurosphere was disrupted and the viral antigen was expressed 5 days after infection (lower). (B) Comparison of viral titers between NSPC and mouse embryonal fibroblasts (MEF) by plaque assay (upper left). Effect of MCMV infection on growth of NSPC at MOI 1 and 10 (upper right). The clonogenic assay was performed NSPC at different MOIs. The cells were infected when one cell per well in 96-well plates and neurospheres were counted at 7 dpi (lower left). MCMV infection inhibits differentiation of NSPC (right lower). Uninfected and MCMV-infected NSPC were induced to differentiate by removing EGF from the medium but adding 2% horse serum. Undifferentiated (green), differentiated (blue) and MCMV-infected differentiated cells (red) were stained with antibodies specific to nestin, GFAP and neurofilament and the quantity of the antigen-positive cells determined by flow cytometric analysis

the differentiation and migration of neural cells, resulting in brain disorders. For these processes, cell-type specificity of viral infection with different infectious dynamics may be associated with pathogenesis [14,60,61], especially viral gene expression during glial and neuronal differentiation.

The infection dynamics of the neural cells in neonatal mouse brains was analysed in mice infected with MCMV in the late stage of gestation. Specific monoclonal antibodies (mAbs) were made to the MCMV IE1 antigen [62] and to the product of the early gene e1 (E1) [63]. The cells expressing the IE1 antigen were mostly localised in the VZ, whereas the cells expressing the E1 antigen were diffusely distributed in the cortex and hippocampus (Figure 4A). The IE1-positive cells were preferentially double-stained with anti-GFAP and anti-nestin antibodies: glial immature cells. These cells are thought to be permissive to infection, because the number of positive cells was similar to that of the viral late gene expressing-cells and viral DNA-positive cells detected by *in situ* DNA–DNA hybridisation. In contrast, the E1-positive cells were double stained with anti-neuron specific enolase (NSE) antibody [62]. This marked contrast in expressing viral antigens in developing brains may depend on differences in the activation of the ie-gene promoters between glial cells and neuronal cells. The infected neuronal cells migrated from the ventricular zone (VZ) to the cortical plate (CP) along with brain development (Figure 4B) [64].

Immediate-early promoter in transgenic mice

Similar to HCMV and other herpesviruses, CMV genes are expressed in three sequential phases: immediate early (IE), early (E) and late (L) [13,15].

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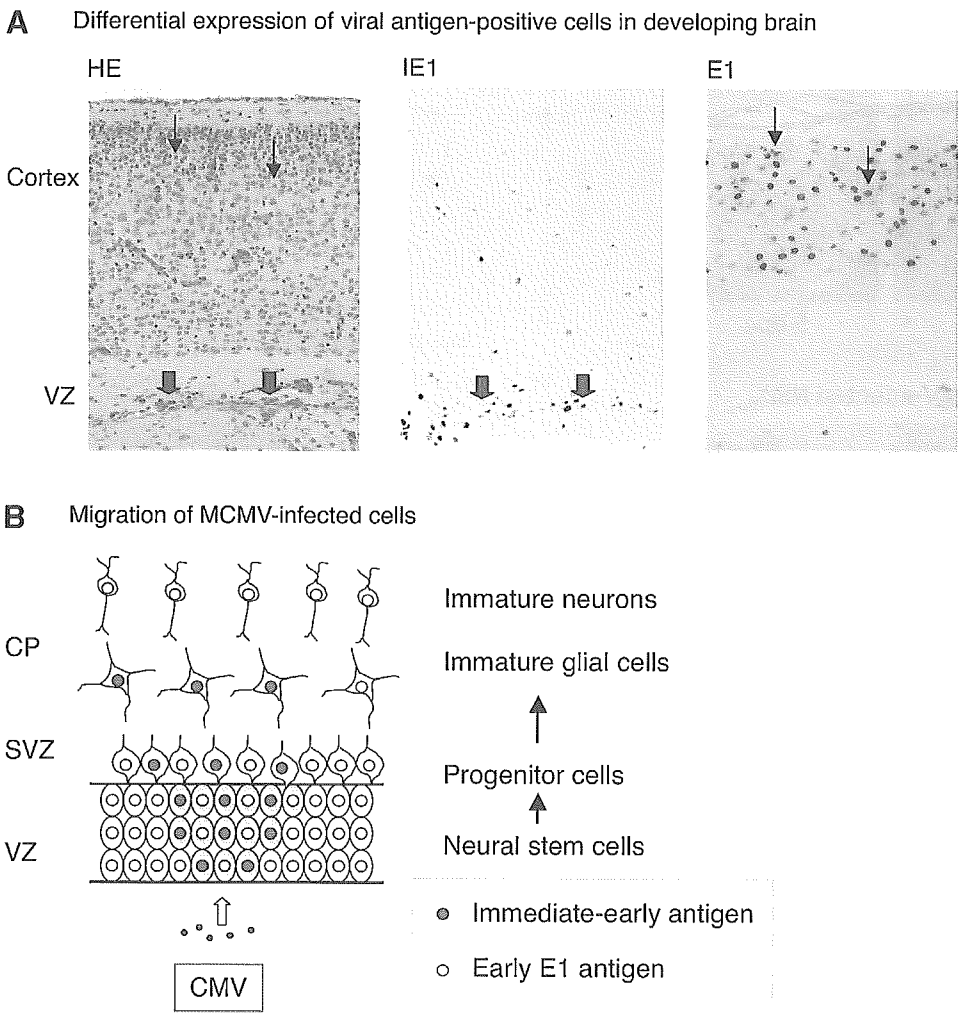

Figure 4. Immunohistochemical comparison between the MCMV IE and E1 antigen-positive cells in developing brains after MCMV infection. (A) The brains were stained with hematoxylin-eosin (HE) (left), with mAbs specific to the IE1 (middle) and E1 antigen (right) in the 7-day-old offspring infected with MCMV on day 15.5 of gestation. (B) Schematic illustration of migration of viral infected-cells from the ventricular zone (VZ) and subventricular zone (SVZ) to cortical plate (CP) during brain development

In HCMV, the most abundantly expressed IE region has at least two immediate-early (ie) genes, called *ie1* (UL123) and *ie2* (UL122), forming a locus that is conserved in other CMVs. MCMV has a predominant IE gene locus that is located in an analogous genomic position to that of HCMV, and is characterised by common structural features (Figure 5A). The two genes that are similar to HCMV *ie1* and *ie2* are referred to as MCMV *ie1* (m123) and *ie3* (M122), respectively [16]. The third gene found in the predominant IE gene, referred to as MCMV ie2 (M128), is transcribed in the opposite direction from the ie1 and ie3 transcripts in this region (Figure 5A) [65]. The MCMV ie2 gene is not related to a HCMV gene, but it is the positional homologue of HCMV UL 127 gene [66].

Expression of IE genes is highly dependent on appropriate cellular transcription factors that bind to the DNA sequence of the CMV major IE enhancer/promoter [67]. However, by DNA transfection, the major IE (MIE) promoter activates heterogenous genes at high levels in most cultured cell lines [68]. Therefore, the MIE promoter has been used as an expression vector. It has been reported that expression of HCMV MIE

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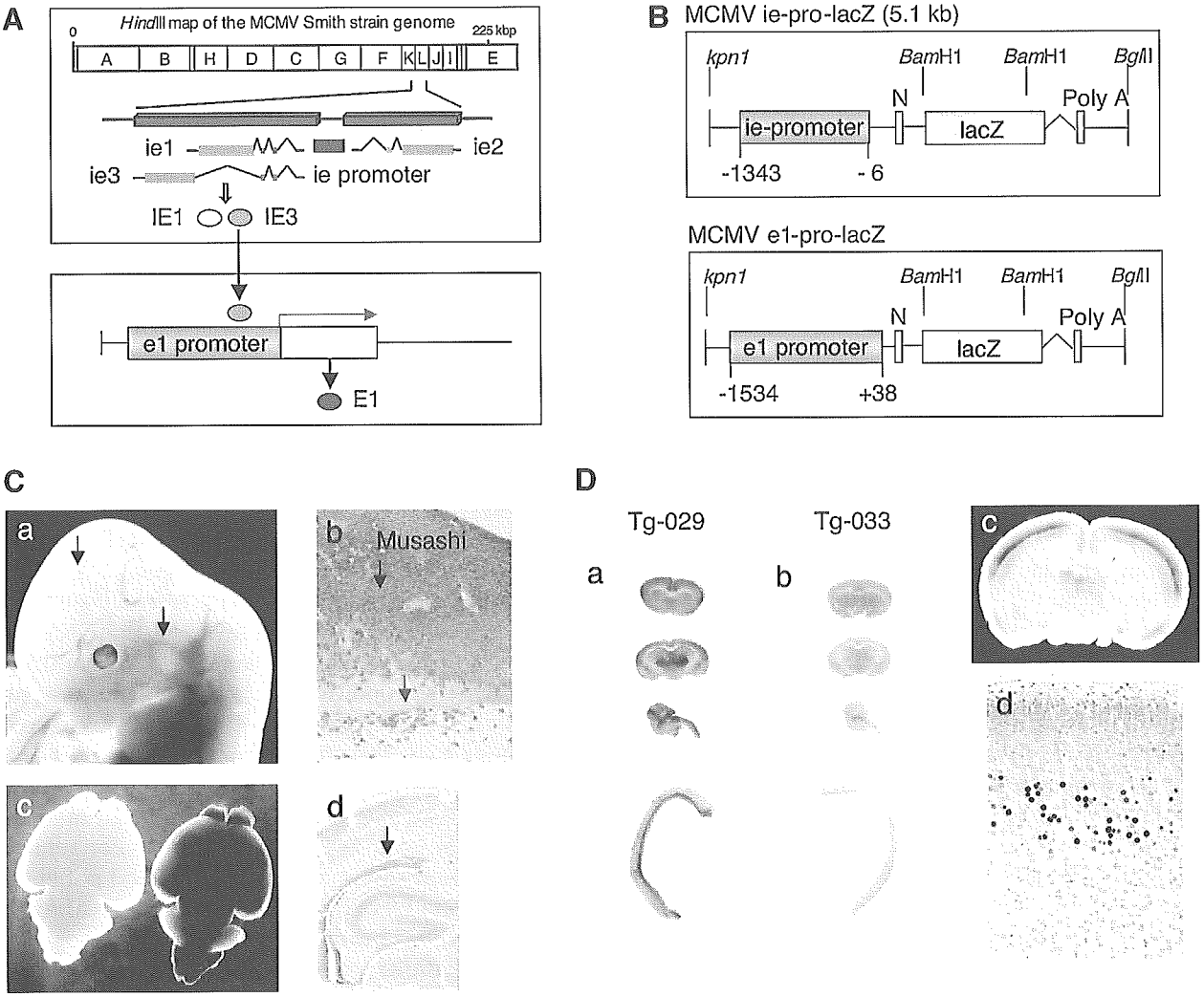

Figure 5. Transgenic mice induced with MCMV ie-promoter (Tg ie-pro) and e1-promoter-lacZ (Tg-e1-pro). (A) Schematic illustration of expression of the MCMV ie1 and ie3 by ie-enhancer/promoter (promoter); the product of ie3 gene (IE3) activates e1-promoter to produce the E1 antigen. (B) The constructs of MCMV ie-promoter-lacz and e1-promoter-lacZ for Tg-ie-pro and Tg-e1-pro, respectively. (C) Activation of β-galactosidase (β-gal) the ie-promoter in vessels (arrows) of the brain of the Tg-ie-pro (E12.5) (a). At this stage, Musashi express in neuroepithelium (black arrow), while the ie-promoter was activated in the endothelium (red arrow) (b). Activation of the ie-promoter was observed in the embryonic brain (E18.5) (c, right), mainly in the ventricular zone (VZ) of lateral ventricle (d, arrow). (D) The transgenic mice with e1-promoter-lacZ (Tg-e1-pro) showed activation of β-gal only in the CNS (Tg-029) (a). β-gal of Tg-033 brain was not activated without MCMV infection (b). Activation of the e1-promoter was observed as layers in cortex (c) and only neuronal cells were stained by immunostaining using anti-β-gal antibody (d)

promoter-lacZ genes was cell-type specific in transgenic mice during embryogenesis [69–71]. In adult tissues, transgene was reported to be expressed in neurons, choroid plexus cells and endothelial cells but not in astrocytes unless the cells were stimulated [70].

Transgenic mice were made with the major IE enhancer/promoter involving nucleotides −1343 to −6 (1338 bp) connected to the reporter gene lacZ (MCMV ie pro1-lacZ) (Figure 5B). In adult mice, transgene expression, as β-galactosidase detected by X-Gal, was detected in the types of cells for which human CMV is tropic such as in distal tubules of the kidney, salivary gland cells, chief cells of the stomach. Interestingly, astrocyte-specific expression of transgene was observed in the brains and in primary glial cultures from the transgenic mice [72]. The spectrum of the expression in organs from MCMV IE promoter was narrow and strictly restricted to particular

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