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Isolation and analysis of an aciclovir-resistant murine cytomegalovirus mutant

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Abstract

An aciclovir (ACV)-resistant murine cytomegalovirus (MCMV) was isolated from the Smith strain and the mutant was analysed. Attempts were also made to identify directly the mutated gene. The 50% inhibitory concentration (IC_{50}) of ACV for the mutant strain was ~30 times higher than that for the wild-type strain. The mutant strain was equally sensitive to ganciclovir (GCV), but slightly resistant to cidofovir (CDV) and foscarnet (PFA) when compared with the wild-type. Molecular analysis of the mutant strain revealed that a single base mutation of cytosine (C) to guanine (G) occurred at the 2476th nucleotide position in the DNA polymerase gene region, resulting in an amino acid substitution of proline (Pro) with alanine (Ala) at codon 826. The marker transfer experiment confirmed that this mutation conferred ACV resistance to MCMV. This mutation at codon 826 was easily identified by means of *Hae* III digestion of the selected PCR product and electrophoresis.

Keywords: Murine cytomegalovirus; Aciclovir; Ganciclovir; Cidofovir; Foscarnet; DNA polymerase; Drug-resistance

1. Introduction

Aciclovir (ACV) has been used to treat patients with herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections. ACV is an acyclic nucleoside analogue and its antiviral activity depends on two virus-coded enzymes, thymidine kinase (TK) and DNA polymerase. The viral TK phosphorylates ACV more efficiently than the cellular TK (Fyfe et al., 1978). As a result, HSV- and VZV- infected cells contain much more ACV triphosphate (ACV-TP) than do uninfected cells (Elion et al., 1977; St. Clair et al., 1980). The ACV-TP then serves as a substrate and inhibits herpesvirus DNA polymerase. ACV-TP inhibits viral DNA polymerases more effectively than cellular DNA polymerases (Martin et al., 1994). Activation by viral TK and selective inhibition of viral DNA polymerase are crucial for the antiviral actions of ACV.

The entire sequence of human cytomegalovirus (HCMV) has been reported (Bankier et al., 1991).

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HCMV was shown not to encode for an enzyme analogous to HSV TK (Zavada et al., 1976). Therefore, ACV is not used for treatment of active HCMV diseases. Murine cytomegalovirus (MCMV) has been utilized as a model for HCMV. As HCMV, MCMV does not encode a viral-specific TK. (Rawlinson et al., 1996). MCMV does not induce TK activity in either TK^+ or TK^- cells resistant to 5-bromodeoxyuridine (BUdR) (Muller and Hudson, 1977; Eizuru et al., 1978; Burns et al., 1981). MCMV does not stimulate TK^- cells to incorporate exogenous thymidine (Muller and Hudson, 1977). Furthermore, the replication of MCMV is not inhibited by BUdR in TK^- cells (Muller and Hudson, 1977; Eizuru et al., 1978; Burns et al., 1981). Thus, MCMV neither induces viral TK activity nor enhances cellular TK activity. Yet, wild-type MCMV shows high susceptibility to ACV in vitro and in vivo (Burns et al., 1981; Wingard et al. 1981; Glasgow et al., 1982).

It is of great interest to know why MCMV is susceptible to ACV even in the absence of TK activity. Therefore, to elucidate the mechanism of this susceptibility, we isolated an ACV-resistant MCMV mutant from the Smith strain and determined the susceptibility of this mutant strain to various antiviral agents. Then we attempted to identify mutation(s) responsible for the ACV resistance of MCMV.

2. Materials and methods

2.1. Antiviral agents

ACV and ganciclovir (GCV) were gifts from Nippon Wellcome, Kobe, Japan. Cidofovir (CDV) was generously provided by Professor E. De Clercq, Leuven University, Belgium. Foscarnet (PFA) was purchased from Sigma, St. Louis, MO.

2.2. Cells and medium

Mouse embryonic fibroblasts (MEF) were prepared by trypsinization of 14–18-day-old mouse embryos and used at the second to fourth passage level for isolation, propagation and titration of MCMV. Balb/c 3T3 cells were used for transfection. MEF were grown in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5% heat-inactivated calf serum (Flow Laboratories, Irvine, VA), 60 μg/ml of kanamycin and 0.12% of sodium bicarbonate. The medium for Balb/c 3T3 cells contained 10% fetal bovine serum instead of 5% calf serum.

2.3. Viruses and isolation of ACV-resistant mutant

The Smith strain of MCMV that was plaque-purified and serially passaged in MEF was used as the wild-type strain. An ACV-resistant MCMV mutant was obtained by the following processes. The plaque-purified wild-type strain of MCMV was passaged in the presence of various concentrations (5, 10, 25 and 50 μM, respectively) of ACV. The MCMV that replicated in the presence of an ACV concentration which yielded $< 20\%$ of the control virus was used for the next cycle of selection using a medium with a higher concentration of ACV. This cycle was repeated up to a final concentration of 50 μM of ACV. The stock samples of both the wild-type and ACV-resistant MCMV were plaque-purified and then filtrated through the membrane filter with a pore size of 450 nm to remove multicapsid or aggregated virions. Finally, stock samples were again plaque-purified twice.

2.4. Titration of viruses

Confluent monolayers of MEF in 35 mm plastic dishes (Sumitomo Ltd., Tokyo, Japan) were inoculated in duplicate with 0.1 ml of serial 10-fold dilutions of MCMV. After virus adsorption for 1 h, the monolayers were overlaid with Eagle's MEM containing 5% heat-inactivated calf serum, 60 μg/ml of kanamycin, 0.12% of sodium bicarbonate and 2.25% methylcellulose, and then incubated at 37°C for 3 days in a humidified atmosphere containing 5% carbon dioxide. The methylcellulose layer was removed and the cells were stained with 0.03% methylene blue. The

number of plaques was evaluated under an inverted microscope.

2.5. Susceptibility test of MCMV strains against antiviral agents

The susceptibility of MCMV strains to antiviral agents was determined by the 50% plaque reduction assay. The monolayers of MEF in 35 mm plastic dishes were inoculated with ~100 plaque-forming units (PFU) of each strain of MCMV. After virus adsorption at room temperature for 1 h, the monolayers were overlaid with Eagle's MEM containing 5% heat-inactivated calf serum, 60 μg/ml of kanamycin, 0.12% of sodium bicarbonate, 2.25% methylcellulose and different concentrations of each antiviral agent. Duplicate dishes were used for each concentration of antiviral agent. The dishes were incubated at 37°C under a humidified atmosphere containing 5% carbon dioxide. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the agent that resulted in 50% plaque reduction.

2.6. Virus growth curve determinations

The monolayers of MEF in culture tubes were inoculated with the Smith strain of MCMV or ACV-resistant strain at a multiplicity of infection (M.O.I.) of 0.2. After adsorption at room temperature for 1 h, the inocula were aspirated off, and 2 ml of Eagle's MEM containing 5% calf serum was added to each culture tube. The infected cells and culture fluid in duplicate tubes were collected every 3 h and stored at −80°C until titration. Then, the viruses were released from the cells by freeze–thawing three times followed by low-speed centrifugation. The supernatant fluid was stored at −80°C until titration. The infectious viruses were plaque-titrated with MEF.

2.7. Preparation of DNA

Viral DNA was prepared according to the Hirt's procedure (Hirt, 1967). The Hirt's supernatant was treated with proteinase K (50 μg/ml) at 56°C for 2 h, followed by phenol: chloroform extraction and ethanol precipitation. Finally, DNA was dissolved in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0: TE) buffer. For marker transfer experiment, wild-type virions of MCMV were prepared as described by Ihara et al. (1994). The viral DNA was treated as mentioned above.

2.8. Amplification of viral DNA fragments by polymerase chain reaction (PCR)

The primers were designed to amplify the fourteen overlapping fragments of viral DNA polymerase coding region and ten overlapping fragments of m97 coding region. Each primer was labelled with biotin at 5′ end for non-radioisotope single strand conformation polymorphism (SSCP) analysis and sequencing. The sequences of the primers for amplifying MCMV DNA polymerase gene are listed in Table 1 and the schematic diagram is shown in Fig. 1. The PCR reaction mixture consisted of viral DNA as template, appropriate primer pairs (1 μM each), deoxyribonucleoside triphosphates (200 μM each), and 1.25 unit of Tth polymerase (Toyobo, Osaka, Japan) in a total volume of 50 μl PCR buffer. The fragment was amplified by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min.

2.9. SSCP analysis and sequencing

For SSCP analysis, PCR product was diluted to 1/10 or 1/100 with a loading buffer consisting of 95% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol. After heating at 90°C for 2 min, 1 μl of the mixture was subjected to gel electrophoresis in 5% polyacrylamide gel containing 5% glycerol as described by Orita et al. (1989). After electrophoretic separation, DNA was transferred to positively charged nylon membrane by capillary blotting and radiated by Imaging high-chemiluminescent detection kit, according to instructions of the Manufacturer (Toyobo). For sequencing, PCR product was purified from agarose gel using QIAX II Extraction Kits (Qiagen GmbH and Qiagen, Hilden, Germany). Sequencing of PCR product was carried out by dideoxy termination method using ΔTth DNA polymerase Sequencing High-Cycle

Table 1
The sequence of the primers defining the amplified MCMV DNA polymerase overlapping DNA fragments

Amplified fragment (Nucleotide No.[a])	Sense primer/Antisense primer 5′-3′
MCMV pol-1 (−29–299)	GGACTCCGATTTCGAG TACTGA
	TTGACGACCTGGTCGT AGGTGT
MCMV pol-2 (181–504)	GAGAAAGAGTACGTG CTC
	TATCGGCGATATCGTA GACG
MCMV pol-3 (439–695)	TACTTCTACTGCGAGA GG
	AAGGGATCCACTCCCA ACT
MCMV pol-4 (612–892)	CAACAACTGGAACAT GTGCC
	CGCTCATACACTCGAT ATCG
MCMV pol-5 (818–1114)	ACATCCTACCGATCGA AG
	CCCGCAACATCTCGTA TT
MCMV pol-6 (1071–1407)	CGTCAAGATCTACACC TTCC
	GGTGTCGAGCTTGTA GTT
MCMV pol-7 (1341–1656)	AGTCGTCCTCGACATG TA
	CAGACAGGTGTAGAT CCT
MCMV pol-8 (1612–1923)	AAGGTCATCTTCGACG GA
	GGAGTAGCACAGATT GTTGG
MCMV pol-9 (1869–2165)	CGATTTCGCCAGTCTG TATC
	CGTTGCACGTTACTTT GAGG
MCMV pol-10 (2115–2412)	GATGATCATGGACAA GCAGC
	CTTGTCGATGCCGTAG AA
MCMV pol-11 (2353–2649)	GTCAAGATCATCTACG GC
	AACCATATGGAGGAC GTC
MCMV pol-12 (2610–2877)	TTTCGTCAAGGCGGTG GTGA
	GGCAAGGTGAGGCAG GTTTT
MCMV pol-13 (2833–3158)	CAAGAGCTCTCCTGCT AT
	ATGTAGATCCTCTGGG GCA
MCMV pol-14 (3070–3324)	GTCACCAACACGCTTA TG
	CAATCGAGATAAGGG AGG

[a] Nucleotide numbers were counted from the start site of the RNA transcript from DNA polymerase gene

Kit (Toyobo). The cycling parameters were as follows: denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, and 30 cycles. The sequencing products were subjected to electrophoresis in 8% polyacrylamide gel containing 8.3 M urea. After electrophoretic separation, the DNA was transferred and irradiated as mentioned above.

2.10. Marker transfer experiments

To confirm the mutation related to ACV resistant, we constructed an ACV-resistant recombinant using marker transfer technique. The DNA fragment containing mutation was amplified by PCR and directly cloned into pCR '2.1 Vector using Original TA Cloning' Kit according to the instructions of the Manufacturer (Invitrogen, Carlsbad, CA). Balb/c 3T3 cells were transfected simultaneously with plasmid DNA and viral DNA using LipofectAMINE PLUS[a] Reagent (Life Technologies, Gaithersburg, MD) according to the instructions of the Manufacturer. After the appearance of cytopathic effect (CPE), the virus was released from the infected cells by three cycles of freeze–thawing and freed of cell debris by low-speed centrifugation. Then, the virus was cultured twice in the presence of 50 μM ACV, and finally plaque-purified twice. The presence of the mutation(s) was confirmed by sequencing.

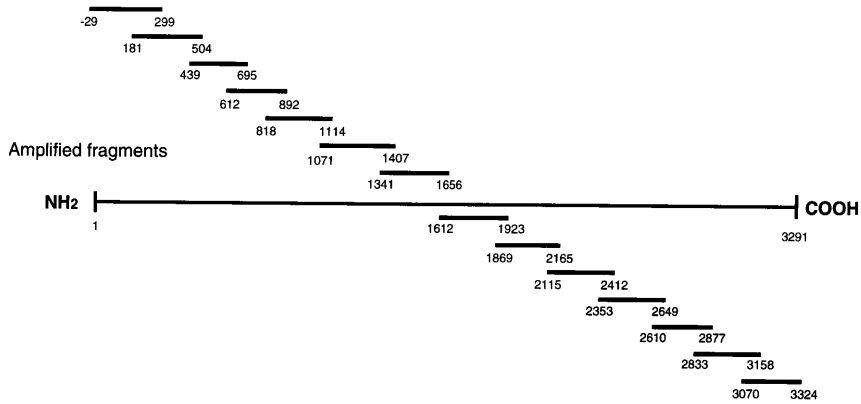

Fig. 1. A schematic representation of the MCMV DNA polymerase region showing the location of the amplified fragments on an approximate amino acid scale. The numbers at intervals down length of PCR products show nucleotide range.

2.11. Restriction enzyme cleavage analysis

PCR product was digested with *Hae* III (Nippon Gene, Toyama, Japan), and subjected to 2.5% agarose gel electrophoresis. The electrophoretic pattern was photographed under UV light.

3. Results

3.1. Isolation and susceptibility of ACV-resistant mutant

An ACV-resistant virus which can replicate in the presence of 50 μM of ACV was derived from the Smith strain of MCMV. This virus was plaque-purified twice and used as the ACV-resistant strain throughout the experiments.

The susceptibility of wild and mutant strain to various antiviral agents was determined by 50% plaque reduction (Table 2). The 50% inhibitory concentration (IC_{50}) of ACV was 90 μM for the mutant, which means that it was ~30 times more resistant than the wild strain. However, the mutant strain was equally sensitive to GCV but slightly resistant to CDV and PFA as compared to the wild-type.

3.2. Molecular analysis of viral DNA polymerase and m97 regions

HCMV encodes a phosphotransferase which phosphorylates GCV. The gene responsible for the enzyme is UL97. MCMV also encodes similar enzyme, and the murine counterpart gene is m97.

The viral DNA polymerase and m97 regions of both wild and mutant strains were compared by PCR-SSCP analysis. Only one fragment pair, nucleotide range from 2353 to 2649 in viral DNA polymerase cording region, showed a different mobility in SSCP analysis (Fig. 2). Then, the region of this fragment was sequenced to identify

Table 2
Susceptibility of ACV-resistant MCMV to various antiviral agents[a]

Antiviral agents	IC_{50} (μM)		Mutant
	Wild strain	Mutant strain	Wild
ACV	3.2	90	28.1
GCV	16	16	1.0
CDV	0.4	0.9	2.3
PFA	85	400	4.7

[a] Each value presents the mean for duplicate determinations from at least two separate experiments.

Fig. 2. PCR-SSCP analysis. The DNA fragments amplified from wild-type MCMV (1) and two cloned ACV-resistant mutants (2–3) were subjected to SSCP analysis.

the mutation(s). When the sequences were compared, the mutant had a single base mutation of cytosine (C) to guanine (G) at nucleotide position 2476, resulting in an amino acid change at codon 826 of proline to alanine (Fig. 3). To examine the possibility of any mutation in m97 region, ten overlapping fragments were also examined by PCR-SSCP analysis. However, there was no difference in the mobility between m97 regions of wild and mutant stains.

3.3. Drug resistance conferred by marker transfer of the mutant DNA to wild-type MCMV

The plasmid DNA containing the mutated viral DNA polymerase region was co-transfected in Balb/c 3T3 cells with the DNA of wild-type MCMV. Recombinant virus was selected twice in the presence of 50 μM ACV. The susceptibility of the recombinant virus to the antiviral agents was the same as that of the ACV-resistant mutant

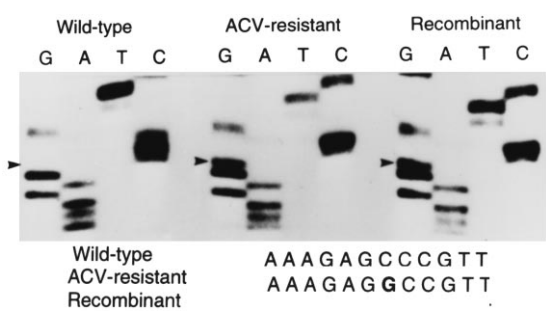

Fig. 3. Sequencing of wild-type, ACV-resistant and recombinant MCMV. ACV-resistant virus and recombinant virus had a single nucleotide substitution at 2476 (C–G).

Table 3
Susceptibility of wild-type MCMV, mutant MCMV and recombinant MCMV to various agents[a]

Antiviral agents	IC_{50} (μM)		
	Wild-type	Mutant	Recombinant
ACV	2.8	82	80
GCV	8.6	9.8	9.6
CDV	0.3	0.8	0.8
PFA	80	340	320

[a] Each value presents the mean for duplicate determinations from two separate experiments.

(Table 3). Median inhibitory concentration of ACV was 80 μM for the recombinant virus.

3.4. Replication of wild-type MCMV, mutant MCMV and recombinant MCMV in MEF

To investigate whether there was any biological difference among the three MCMV strains, the growth curves of these viruses in MEF were compared. Amounts of virus produced every 3 h during the virus life cycle were quantified by plaque titration. Overall, there was no marked difference in the time course of replication or in total virus yield among wild-type, mutant and recombinant MCMV strains (data not shown).

3.5. Identification of mutation site by PCR and restriction fragment length polymorphism (RFLP)

Identification of the mutation at codon 826 was accomplished by means of RFLP of the selected PCR product in the nucleotide range from 2353 to 2649. This region in wild strain contained one GGCC recognition site for *Hae* III. In the ACV-resistant strain, however, the sequence GCCC had changed to GGCC, which produced an additional recognition site for *Hae* III. Consequently, the codon 826 CCC (proline) had changed to GCC (alanine). The PCR products amplified from wild and mutant strains were digested with *Hae* III and subjected to 2.5% agarose gel electrophoresis. The PCR product from wild strain produced two bands, 76 and 221 bps. In contrast, the PCR product from the mutant strain produced three

bands, 76, 48 and 173 bps as shown in Fig. 4 (A–B). Therefore, the mutation was easily detected by digestion with *Hae* III followed by electrophoresis.

4. Discussion

Human cytomegalovirus (HCMV) causes serious and often life-threatening diseases in fetuses, neonates and immunocompromised hosts, such as recipients of organ transplants or patients with malignancies or the acquired immune deficiency syndrome (AIDS) (Britt and Alford, 1996). Currently, inhibitors of viral DNA synthesis are available as anti-CMV drugs. These include GCV, PFA and CDV.

Unfortunately, no animal model is available for studying antiviral agents against HCMV in vivo because of its strict species-specificity. MCMV has been well investigated as an animal model for evaluating anti-CMV compounds. MCMV is susceptible to GCV, PFA and CDV (Smee et al., 1992, 1995; Okleberry et al., 1997; Duan et al. 1998); however, it is also sensitive to ACV (Burns et al., 1981; Wingard et al., 1981; Glasgow et al., 1982). The susceptibility of MCMV to ACV may reside in the DNA polymerase rather than the phosphotransferase, as mutants resistant to ACV also show less susceptibility to chemicals that

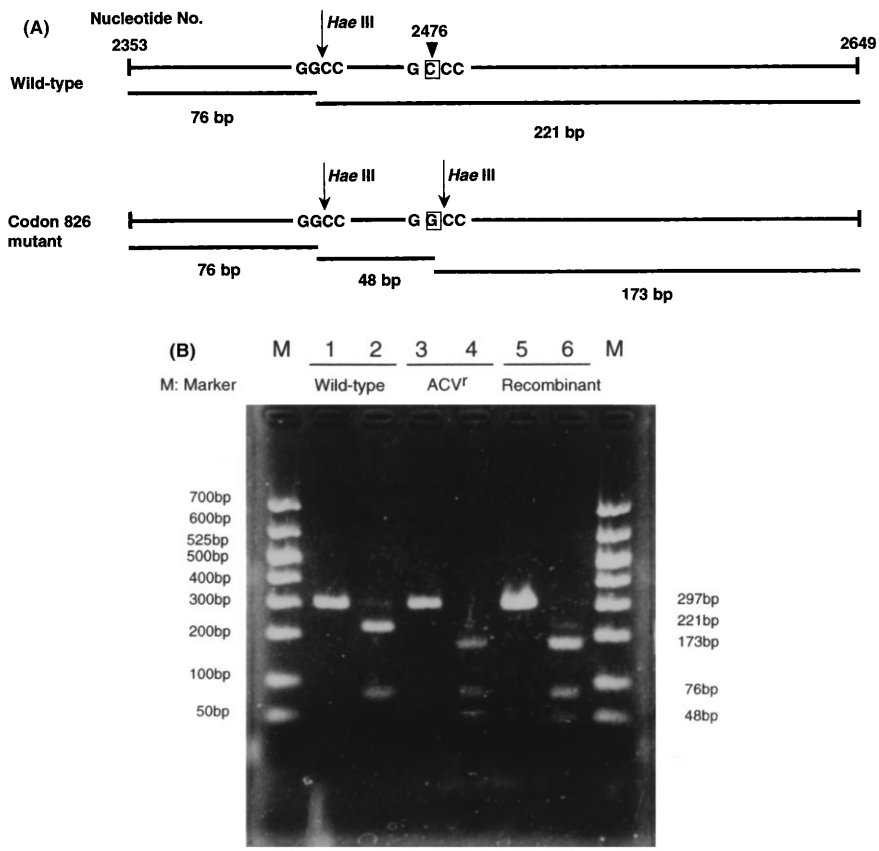

Fig. 4. Rapid detection of the mutation at codon 826. (A) Schematic representation of *Hae* III cleavage sites of wild and mutant strains. Digestion of wild-type virus DNA resulted in one cleavage, represented by two DNA fragments in the gel. Digestion of mutant DNA gave two cleavages, resulting in three fragments. (B) The selected PCR products from wild-type (1–2), ACV-resistant mutant (ACV^r) (3–4) and recombinant viruses (5–6) were digested with *Hae* III (2, 4 and 6) or untreated (1, 3 and 5). They were subjected to 2.5% agarose gel electrophoresis.

interact with DNA polymerase such as phosphonoacetic acid (PAA) (Burns et al., 1982; Sandford et al., 1985) and PFA (Elliott et al., 1991).

The analysis of ACV-resistant mutant may offer a clue to help elucidating the mechanism of the anti-MCMV effect of ACV. We isolated and characterized an ACV-resistant mutant of MCMV from the Smith strain. Drug susceptibility tests showed that the ACV-resistant mutant was equally sensitive to GCV but slightly resistant to CDV and PFA, when compared with the wild-type. As this ACV-resistant mutant showed cross-resistance to PFA, its DNA polymerase gene seemed to be the site of mutation. In fact, molecular analysis of the mutant revealed a novel mutation in the DNA polymerase gene, namely, a single base substitution (from C to G) at the 2476th nucleotide position in the DNA polymerase region, with consequently an amino acid substitution, from proline to alanine, at codon 826. It was confirmed by marker transfer experiment that this mutation conferred ACV resistance to MCMV.

The amplified fragment which contains the mutation was detectable by SSCP analysis. For rapid and direct identification of MCMV responsible for ACV-resistance, we employed RFLP analysis. SSCP followed by restriction enzyme cleavage provides a rapid sensitive detection of certain mutations in PCR-amplified DNA fragments. In our case, the mutation at codon 826 (CCC to GCC mutation) was detected by *Hae* III digestion (recognition sequence GGCC) and electrophoresis. However, this identification is specific only for CCC to GCC mutation. If the mutation were not CCC to GCC, of course, other endonuclease enzymes would be required to recognize the mutation site.

Incidentally, no mutation could be found in the m97 gene that is homologous to HCMV UL97 (phosphotransferase). However, in this study both wild and mutant strains of MCMV were less susceptible to GCV than in previous reports (Smee et al., 1995; Okleberry et al., 1997).

Overall, there is much homology between MCMV and HCMV DNA polymerase gene. The amino acid sequences revealed that 576 of the 1097 amino acids of the MCMV DNA polymerase have an identically matched residue in the HCMV DNA polymerase (Elliott et al., 1991). In addition, the amino acid residue Pro826 of MCMV DNA polymerase, shown to be responsible for ACV susceptibility of MCMV in this study, is conserved in the DNA polymerase of HCMV (Pro945) and rhesus monkey CMV (Pro792) (Swanson et al., 1998). This position of Pro in HCMV polymerase is located in the gap between I and VII conservative regions. However, a mutation at the homologous position to the Pro826 of MCMV has not been reported in the human or rhesus CMV DNA polymerase yet. In addition, there is no reduction in growth fitness of the MCMV mutant encoding the Pro826Ala mutation.

Antiviral effect of ACV requires initial phosphorylation of ACV to form ACV monophosphate and final inhibition of viral DNA synthesis by ACV-TP. This investigation showed that the DNA polymerase may at least be one of the target enzymes for the anti-MCMV action of ACV. This was evidenced by the fact that the ACV-resistant mutant had a mutation in the DNA polymerase gene. The results described in this paper are compatible with those of a previous paper (Sandford et al., 1985). We have already reported on the role of MCMV DNA polymerase in the antiviral activity of ACV in vitro (Ochiai et al., 1992). In that study, we partially purified the DNA polymerase and determined its kinetic constants for ACV triphosphate. We raised a possibility that MCMV DNA polymerase might be highly sensitive to ACV triphosphate. However we did not use an ACV-resistant mutant at that time.

MCMV does not encode for a TK that phosphorylates ACV (Eizuru et al., 1978; Rawlinson et al., 1996). However, the HCMV UL97 homologue in MCMV genome, m97, has been identified (Rawlinson et al., 1997). In fact, ACV is phosphorylated by UL97 protein, although to a lesser degree than GCV (Zimmermann et al., 1997). It is thus conceivable that this m97 gene product may be responsible for phosphorylation of ACV and for the susceptibility of MCMV to ACV. Unfortunately, isolation of a m97 mutant has not been achieved so far. This means that the exact function of the m97 gene remains to be elucidated. To elucidate the exact mechanism by which ACV inhibits MCMV replication, the phosphorylation

pattern of ACV in MCMV-infected cells should be addressed in future studies.

References

Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Chee, M.S., Hutchinson, C.A., Kouzarides, T., Martignetti, J.A., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M., Barrell, B.G., 1991. The DNA sequence of the human cytomegalovirus genome. DNA Seq. 2, 1–12.

Britt, W.J., Alford, C.A., 1996. Cytomegalovirus. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, third ed. Lippinocott-Raven, Philadelphia, pp. 2493–2523.

Burns, W.H., Wingard, J.R., Bender, W.J., Saral, R., 1981. Thymidine kinase not required for antiviral activity of acyclovir against mouse cytomegalovirus. J. Virol. 39, 889–893.

Burns, W.H., Wingard, J.R., Sandford, G.R., Bender, W.J., Saral, R., 1982. Acyclovir in mouse cytomegalovirus infections. Am. J. Med. 73, 118–124.

Duan, J., Paris, W., Kibler, P., Bousquet, C., Liuzzi, M., Cordingley, M.G., 1998. Dose and duration-dependent of ganciclovir treatment against murine cytomegalovirus infection in severe combined immunodeficient mice. Antiviral Res. 39, 189–197.

Eizuru, Y., Minamishima, Y., Hirano, A., Kurimura, T., 1978. Replication of mouse cytomegalovirus in thymidine kinase-deficient mouse cells. Microbiol. Immunol. 22, 755–764.

Elion, G.B., Furman, P.A., Fyfe, J.A., De Miranda, P., Beauchamp, L., Schaeffer, H.J., 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)-guanine. Proc. Natl. Acad. Sci. USA 74, 5716–5720.

Elliott, R., Clark, C., Jaquish, D., Spector, D.H., 1991. Transcription analysis and sequence of the putative murine cytomegalovirus DNA polymerase gene. Virology 185, 169–186.

Fyfe, J.A., Keller, P.M., Furman, P.A., Miller, R.L., Elion, G.B., 1978. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J. Biol. Chem. 253, 8721–8727.

Glasgow, L.A., Richards, J.T., Kern, E.R., 1982. Effect of acyclovir treatment on acute and chronic murine cytomegalovirus infection. Am. J. Med. 73, 132–137.

Hirt, B., 1967. Selective extraction of polyoma DNA from infected mouse cell culture. J. Mol. Biol. 26, 365–369.

Ihara, S., Takekoshi, M., Mori, N., Sakuma, S., Hashimoto, J., Watanabe, Y., 1994. Identification of mutation sites of a temperature-sensitive mutant of HCMV DNA polymerase. Arch. Virol. 137, 263–275.

Martin, J.L., Brown, C.E., Matthews-Davis, N., Reardon, J.E., 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. 38, 2743–2749.

Muller, M.T., Hudson, J.B., 1977. Thymidine kinase activity in mouse 3T3 cells infected by murine cytomegalovirus. Virology 80, 430–433.

Ochiai, H., Kumura, K., Minamishima, Y., 1992. Murine cytomegalovirus DNA polymerase: purification, characterization and role in the antiviral activity of acyclovir. Antiviral Res. 17, 1–16.

Okleberry, K.M., Warren, R.P., Smee, D.F., 1997. Metabolism of ganciclovir and cidofovir in cells infected with drug-resistant and wild-type strains of murine cytomegalovirus. Antiviral Res. 35, 83–90.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., Sekiya, T., 1989. Detection of polymorphism of human DNA by gel electrophoresis as single-strand conformation polymorphism. Proc. Natl. Acad. Sci. USA 86, 2766–2770.

Rawlinson, W.D., Farrell, H.E., Barrell, B.G., 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. J. Virol. 70, 8833–8849.

Rawlinson, W.D., Zeng, F., Farrell, H.E., Cunningham, A.L., Scalzo, A.A., Booth, T.W., Scott, G.M., 1997. The murine cytomegalovirus (MCMV) homolog of the HCMV phosphotransferase (UL97(pk)) gene. Virology 233, 358–363.

Sandford, G.R., Wingard, J.R., Simons, J.W., Staal, S.P., Saral, R., Burns, W.H., 1985. Genetic analysis of the susceptibility of mouse cytomegalovirus to acyclovir. J. Virol. 54, 104–113.

Smee, D.F., Morris, J.L.B., Leonhardt, J.D., Mead, J.R., Holy, A., Sidwell, R.W., 1992. Treatment of murine cytomegalovirus infection in severe combined immunodeficient mice with ganciclovir, (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine, interferon, and bropirimine. Antimicrob. Agents Chemother. 36, 1837–1842.

Smee, D.F., Barnett, B.B., Sidwell, R.W., Reist, E.J., Holý, A., 1995. Antiviral activities of nucleosides and nucleotides against wild-type and drug-resistant strains of murine cytomegalovirus. Antiviral Res. 26, 1–9.

St. Clair, M.H., Furman, P.A., Lubbers, C.M., Elion, G.B., 1980. Inhibition of cellular α and virally induced deoxyribonucleic acid polymerases by the triphosphate of acyclovir. Antimicrob. Agents Chemother. 18, 741–745.

Swanson, R., Bergquam, E., Wong, S.W., 1998. Characterization of rhesus cytomegalovirus genes associated with antiviral susceptibility. Virology 240, 338–348.

Wingard, J.R., Bender, W.J., Saral, R., Burns, W.H., 1981. Efficacy of acyclovir against mouse cytomegalovirus in vivo. Antimicrob. Agents Chemother. 20, 275–278.

Zavada, V., Erban, V., Rezacova, D., Vonka, V., 1976. Thymidine-kinase in cytomegalovirus infected cells. Arch. Virol. 52, 333–339.

Zimmermann, A., Michel, D., Pavić, I., Hample, W., Lüske, A., Neyts, J., De Clercq, E., Mertens, T., 1997. Phosphorylation of aciclovir, ganciclovir, penciclovir and S2242 by the cytomegalovirus UL97 protein: a quantitative analysis using recombinant vaccinia viruses. Antiviral Res. 36, 35–42.