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Intracellular uptake of thymidine and antiherpetic drugs for thymidine kinase-deficient mutants of herpes simplex virus type 1

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Abstract

The influence of the thymidine (Thd) kinase (TK) of herpes simplex virus type 1 (HSV-1) on the intracellular uptake and anabolism of nucleosides has been investigated. To compare the differences between the TK-positive (TK^+) and TK-deficient strains, acyclovir (ACV)-resistant strains were cloned from a cell culture and classified into 2 groups, viz. the TK-partial (TK^p) and TK-negative (TK^-). The cellular uptake of thymidine was highly dependent on the viral TK (vTK) activity. The TK^+ strain showed the highest level of intracellular thymidine uptake, the TK^p strain a moderate level, which varied from strain to strain, and the TK^- and mock strains showed little uptake. The inhibition of viral replication by ACV, ganciclovir (GCV) and penciclovir (PCV) did not decrease the Thd uptake at all. On the contrary, a notable increase found to be induced by ACV. The influence of the vTK on the uptake of GCV or PCV was much greater than that of ACV. The metabolism was generally less dependent on the vTK activity than the influx. The influx and phosphorylation rates of GCV and PCV were dependent on the substrate specificity of the vTK.

Keywords: Herpes simplex virus; Thymidine kinase; TK-deficiency; Drug-resistance; Antiherpetic drug; Nucleoside uptake; Nucleoside anabolism

1. Introduction

The thymidine (Thd) kinase (TK)-encoded herpes simplex virus type 1 (HSV-1) is a pyrimidine salvage enzyme responsible for the synthesis of thymidine monophosphate (TMP) from Thd and ATP. The HSV-1 TK possesses not only Thd kinase activity but also deoxycytidine (dCyd) kinase (dCK) activity, which phosphorylates dCyd to dCyd monophosphate (Jamieson et al., 1974; Thouless and Wildy, 1975), and thymidylate kinase (TMPK) activity, which phosphorylates TMP to TDP (Chen and Prusoff, 1978; Kit et al., 1974; Maga et al., 1994). Furthermore, the viral TK (vTK), because of its low substrate specificity, can also phosphorylate to mono-and/or di-phosphate forms a wide variety of pyrimidine and purine nucleoside analogues (De Clercq, 1982; Fyfe et al., 1978; Fyfe, 1982). The vTK catalyzes the initial activation (phosphorylation) of antiviral nucleoside analogues such as acyclovir (ACV), ganciclovir (GCV), penciclovir (PCV), and many 5-substituted deoxyuridine compounds. The vTK consists of a homodimeric complex of the UL23 protein product, whose structure has been successfully elucidated (Brown et al., 1995).

Intensive studies involving the vTK have been performed, especially in the area of antiviral resistance (Bacon et al., 2003; Chibo et al., 2004; Gilbert et al., 2002; Sarisky et al., 2003) and chemo/gene therapy (Balzarini et al., 1993; Degrève et al., 2000a; Fillat et al., 2003) with the goal of selectively killing genetically modified cancer cells with antiherpetic drugs. Most of the reported cases of antiviral resistance to vTK-dependent antiherpetic compounds were due to TK-deficiency and very rarely to a change in the substrate specificity of vTK or viral DNA polymerase activity. The development of drug-resistant and site-directed mutants (Degrève et al., 2000b; Kussmann-Gerber et al., 1998) has been helpful in identifying some regions involved in substrate binding. Recent intensive studies on the clinical isolates of HSV to ACV (Chibo et al., 2004) or PCV (Sarisky et al., 2003) have provided structural information which is helpful in designing vTK molecular models.

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The roles of the vTK in viral replication and pathogenesis, such as virulence and reactivation from the latent state (Chen et al., 2004; Coen et al., 1989; Efstathiou et al., 1989; Griffiths et al., 2003; Harris et al., 2003; Thompson and Sawtell, 2000) still remain to be elucidated. Mainly on the basis of TK-mutant studies, it is considered that TK-deficient mutants are less virulent and show less reactivation from latency. However, little difference has been observed between TK-positive (TK^+) and TK-deficient HSV in cell culture systems (Tenser et al., 1981). A 25- to 50-fold expansion of the dTTP pool in HSV-1-infected BHK C13 cells and mutant cells lacking both cellular TK (cTK) and dCK activities was reported (Jamieson and Bjursell, 1976). It was also reported that the addition of Thd enhanced the viral reactivation in an explant culture, suggesting that the Thd levels were more important than the general nucleotide pool imbalance (Tenser et al., 1996) and that the cTK activity could compensate for the vTK activity in the process of viral reactivation from the latent state (Chen et al., 1998). The influence of the vTK on the intracellular levels, uptake (or influx) and anabolism of nucleosides might be important to elucidate its biological functions.

The aims of this study were as follows: (1) to clarify whether there are any common features among the TK-deficient mutants in terms of the intracellular uptake of Thd and its incorporation into DNAs; (2) to investigate the influence of vTK-dependent drugs such as ACV, GCV and PCV on Thd uptake; (3) to determine whether the recombinant TK from HSV-1 strain F, expressed constituently in cTK-deficient (cTK^-) cells, could compensate for the features found in TK-deficient mutants; and (4) to examine the influence of the vTK activity on the influx and anabolism of ACV, GCV and PCV.

2. Materials and methods

2.1. Cells and viruses

Vero (African green monkey kidney), 143B (human osteosarcoma) and HSV-1 strain F (F) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The FTK143B cell line (Kim et al., 2002) constitutively expressing the vTK gene was cloned after the transfection of 143B cells with a eukaryotic expression vector containing the TK gene of F. HSV-1/AR1–AR9 (AR1–AR9) were laboratory-derived TK-deficient ACV-resistant (ACV^r) mutants of HSV-1/F (Kim et al., 2002). Each cell line was maintained with different supplements. For the Vero cells, Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaitherburg, MD) with 5% fetal bovine serum (FBS) (Life Technologies) (DMEM/5% FBS) was used. For the 143B cells, 15 μg/ml 5-bromo-2′-deoxyuridine (BrdUrd) (Sigma, St. Louis, MO) was added to DMEM/10% FBS to suppress the cTK^+ cells. For the FTK143B cells, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) (Sigma) and 600 μg/ml G418 (Sigma) were added to DMEM/10% FBS. For the virus infection, all of the cell lines were grown in the absence of BrdUrd, HAT and G418 for two passages prior to the experiment. Vero cells were used for the stock virus preparation.

2.2. Compounds

PCV was kindly provided by the former SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK. GCV was obtained from Hoffmann La Roche, Basel, Switzerland in the form of the sodium salt. GCV was dissolved in DMEM at a concentration of 20 mg/ml, ACV (Sigma) and PCV were dissolved in dimethylsulfoxide at the same concentration and all three were stored at −20 °C as stock solutions. Working dilutions were prepared in the assay medium, DMEM/2% FBS, just prior to use. [^{14}C]Thd, [2′-^{3}H]-ACV ([^{3}H]ACV) and [methyl-^{3}H]-Thd ([^{3}H]Thd) were purchased from NEN Life Science (Boston, MA), Sigma and Amersham (Little Chalfont, Buckinghamshire, UK) and their specific activities were 59.7, 18.3, and 85 Ci/mmol, respectively. [8-^{3}H]-GCV ([^{3}H]GCV) and [8-^{3}H]-PCV ([^{3}H]PCV) were purchased from Moravek Biochemicals (Brea, CA); their specific activities were 20 and 14.9 Ci/mmol, respectively.

2.3. Antiviral evaluation

Each virus strain was subsequently tested for its in vitro susceptibilities to a broad range of antiviral compounds by means of a cytopathic effect (CPE) inhibition assay in Vero cells. Confluent cells grown in 96-well microplates (at 3–4 days after seeding) were infected with the different virus strains at an inoculum size of 100 $CCID_{50}$ per well. The inoculum sizes were verified by a simultaneous CPE assay. After 1 h of incubation, the residual virus was removed and 100 μl of the drugs diluted with DMEM/2% FBS was applied to the infected cells in duplicate. The cells were incubated at 37 °C for 3 days. The antiviral activity in the virus-infected cell cultures and the cytocidal effects of the drugs in the mock-infected cells were measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Kim et al., 2002), as well as by microscopic observation. The antiviral activity of the drugs at the dose achieving 50% cell survival of the cell cultures against the CPE of the virus strains was defined as the 50% effective concentration (EC_{50}).

2.4. Nucleotide sequencing

The genomic DNAs of strain F were digested with *Bam* HI and the 3.4 kb fragment containing the TK gene was introduced into pBluescript (Stratagene, La Jolla, CA). An approximately 1.9 kb fragment of the TK gene of each mutant was amplified directly from whole DNAs isolated from virus-infected cell lysates using Taq polymerase. The PCR products were ligated with pGEM-T vector (Promega, Madison, WI). The nucleotide sequence of the plasmids containing the TK gene was determined by the PCR-directed sequencing method. The overlap between sequencing runs was evaluated to allow for the identification of mutations within the primer sequence.

2.5. Cloning of the TK gene for E. coli expression system

HSV-1 DNAs were prepared directly from viruses produced after infecting Vero cell cultures with the variants. The viral

DNAs from the different strains were digested with *Bam* HI and the 3.4 kb fragment including the TK gene was purified by electrophoresis in agarose gels. The open reading frame (ORF) region of the TK gene was inserted into the *Bam* HI and *Eco* RI sites of the plasmid pGEX-4T-1 (Amersham Bioscience, Piscataway, NJ) for the expression of the TK gene in *Escherichia coli* (*E. coli*). The glutathione sepharose transferase (GST)-fused recombinant vTK (recTK) proteins were purified by means of a glutathione sepharose 4B (Amersham Bioscience) column. Where necessary, GST was cleaved from the recTK by thrombin-digestion and removed using a GST column.

2.6. Analysis of vTK activity

Confluently grown cells or HSV-1-infected Vero cells showing 50% CPE were harvested, washed twice with calcium-and magnesium-free phosphate-buffered saline [PBS(−)] and resuspended in PBS(−)/3 mM DTT. The vTK activity was measured using the cell lysates, GST-removed recTK proteins and recTK proteins. The cells were treated with 0.1% of Nonidet P-40 (NP-40) and then incubated on ice for 5 min before extraction. Following centrifugation for 2 min, the supernatant was stored at −70 °C until the enzyme assay. 40 μl of the enzyme reaction buffer (20 mM phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 5 μM [^{3}H]Thd, 10 mM ATP, 10 mM DTT, 10 μM thymidine) was mixed with various concentrations of the proteins. The mixture was reacted for 30 min at 37 °C and filtered through a Whatman DE81 filter and washed twice with ice-cold 4 mM ammonium formate and twice with ethanol. The radioactivity of the dried filters was measured using a liquid scintillation counter (LS 6000TA, Beckman Instruments, Palo Alto, CA).

2.7. Plaque autoradiography

Plaque autoradiography was performed by infecting cTK$^-$ cells with HSV-1 (Tenser et al., 1983). The serial dilutions of each viral strain were inoculated onto 143B cell monolayers formed on cover glasses in 6-well plates and the cells were cultivated for 3 days at 37 °C. The medium in the wells showing well separated plaques of sufficient number was removed and the monolayer was incubated with 0.2 μCi of [^{14}C]Thd in 1.5 ml DMEM/2% FBS for 4 h at 37 °C. The cells were washed with PBS(−) twice for 15 min at 37 °C each time. The cells were then stained with crystal violet, washed, and air-dried. The plates were placed in contact with an X-ray film (Fuji Co., Tokyo, Japan) for 5 days at −70 °C and developed in the dark room.

2.8. Preparation of cell extracts for nucleoside and nucleotide analysis

Confluent Vero, 143B and FTK143B cells seeded in 100-mm Petri dishes were infected with the various strains of HSV-1 at 5–10 p.f.u. per cell. At 1 h postinfection (p.i.) the excess inoculum was replaced with 2 ml of fresh medium containing 2% FBS and a radioactive chemical (1 μM/dish). After incubation at 37 °C for 8 h, the medium was removed and the cells were washed twice with 10 ml of ice-cold PBS(−). The cells were dislodged with a sterile rubber policeman and transferred into tubes stored in an ice-bath. The harvested cells were washed twice by centrifugation. The cell pellets were stored at −20 °C for 12 h. Six hundred microliters of cold 0.5 M perchloric acid was added to each tube, which was then vortexed and stored for 30 min at 4 °C. The precipitate was discarded after centrifugation at 12,000 × *g* for 5 min. The supernatant (acid-soluble extract) was neutralized with 150 μl 4 M KOH and buffered with 1 M potassium phosphate buffer at pH 7.2, centrifuged and the supernatant was evaporated under reduced pressure. The residue containing the nucleosides and nucleotides was dissolved in 1 M potassium phosphate buffer and stored at −20 °C.

2.9. Nucleoside and nucleotide analysis

The acid-soluble extracts were analyzed with a high-pressure liquid chromatography (HPLC) (Class VP-series LC10AT, Shimadzu, Kyoto, Japan) fitted with a hypersil BDS 4.6/25 cm column (Thermoquest, Runcorn, UK). A linear gradient of 5 mM tetrabutylammonium bromide in 20 mM K_2HPO_4 at pH 3.5 (buffer A) to buffer A and 60% acetonitrile at pH 3.5 (buffer B) was used to separate the metabolites as follows: 5 min of 100% buffer A, 40 min of a linear gradient to 70% buffer B, 10 min of 100% buffer B, 5 min of a linear gradient to 100% buffer A, and 5 min of equilibration with buffer A. The flow rate was 1 ml/min. The ultraviolet absorption of the eluate was monitored at 254 nm. Fractions (1.0 ml) were collected every 1 min, and the radioactivity in each fraction was measured by liquid scintillation spectrometry.

2.10. Measurement of the uptake and incorporation of nucleoside derivatives into DNA

Confluent Vero cells seeded in 96-well plates were infected with the various strains of HSV-1 at 10 p.f.u. per cell, and incubated for 1 h at 37 °C. The culture fluid was removed, and the cells washed twice with the assay medium. The radiolabeled nucleosides were added (0.5 μCi/well) and incubation allowed to continued. At various times thereafter and in triplicate, the medium was removed by aspiration and the cells were washed twice with PBS(−). The cells in each well were dislodged by 50 μl of trypsin-EDTA and transferred to tubes in an ice bath. The remaining cells in each well were collected with 100 μl of PBS(−) and combined with the contents of each tube, which were then stored at −70 °C overnight. The tubes were thawed and 50 μl of 50% trichloroacetic acid (TCA) and 100 μl of 10% TCA (total 300 μl liquid/tube) were added. For the uptake assay, 10 μl of each crude cell lysate was removed and spotted on a Whatman filter paper and dried. After 30 min on ice, the TCA precipitates (acid-insoluble fraction) resulting from the remaining 290 μl of the lysate were collected on a GF/C filter (Whatman, Maidstone, Kent, UK) and washed with 10% TCA and ethyl alcohol. The radioactivity of 10 μl of the crude lysate and acid-insoluble fractions (nucleic acids and proteins) were determined with the scintillation counter. The total uptake was calculated by multiplying the count of the 10 μl-fraction on the Whatman filter by 30 and the total DNA incorporation by multi-

plying the count of the TCA-precipitated 290 μl-fraction on the GF/C filter by 300/290.

2.11. Statistics

The statistical evaluations of the experiments were performed by ANOVA followed by a Newman–Keuls multiple comparison test and the results were expressed in the form of the arithmetic mean ± standard deviation of the mean (S.D.). A value of $P < 0.05$ was considered significant in all cases.

3. Results

3.1. Characterization of antiviral-resistant HSV

We isolated the 9 ACV^r F clones which appeared in the presence of 10–25 μg/ml of ACV, performed plaque purification three-times and named these nine clones, AR1–AR9, respectively. We previously reported on both the antiviral activity and cytotoxicity, as well as the presence of mutations, in the ORF of the TK gene of AR1 and AR2 (Kim et al., 2002). The EC_{50} values of the drugs for each strain measured by the CPE inhibition assay at 3 days p.i. are shown in Table 1. The mutants showed a greater than 30-fold resistance to ACV. However, their sensitivities to GCV and PCV were markedly different. Their vTK activities were measured with virus-infected Vero cell lysates and also with recTK proteins produced in *E. coli*. The extracts from the F-infected cells showed dose-dependent enzyme activities, but those of the mutant virus-infected cells showed only a low level of vTK activity similar to that of the mock-infected cells (Fig. 1A). All of the mutants were considered to be TK-deficient. To produce recTK proteins in *E. coli*, the ORF of the TK gene was inserted into the plasmid, pGEX-4T-1, leading to the production of GST-fused TK proteins. Fig. 1B shows the vTK activity of the GST-removed recTK proteins of F, AR1, AR2, and AR7. AR2 had a much lower vTK activity than F, but its activity was dose-dependent, whereas AR1 and AR7 showed no enzyme activity at protein concentrations of up to 100 μg/ml. The vTK activities of the other mutant strains were measured with the GST-fused vTK proteins by varying the volume of the enzyme (Fig. 1C). Although the amount of protein used varied, AR2, AR4, AR5, AR6, and AR9 showed much lower though still significant levels of activity compared to F. AR3 and AR8 had no activity. Plaque autoradiography was performed with [^{14}C]Thd as the enzyme substrate at 3 days p.i. (Fig. 2). AR1, AR3, AR7, and AR8 failed to form labeled plaques. AR2, AR4, AR5, AR6, and AR9 showed labeled plaques similar to that of strain F. Using the data obtained from the enzyme assay with the recTK proteins and plaque autoradiography, we classified the TK-deficient mutants into two groups, viz. vTK$^-$ and vTK^p. Those viral strains (AR1, AR3, AR7, and AR8) with no enzyme activity were assigned to the vTK$^-$ group, while AR2, AR4, AR5, AR6, and AR9, which had some enzyme activity, were assigned to the vTK^p group.

The presence of mutations in the ORF of their TK genes was then determined as described in Section 2 (Table 1). All of them except for AR3 showed one or two nucleotide substitutions causing at least one amino acid substitution. AR3 and AR8 showed one base deletion mutation causing frameshifted elongation or prematuration of the TK polypeptide, respectively. AR6 had an additional mutation, i.e. A719V, in the conserved region II of the DNA polymerase gene, UL30. The mutations

Table 1
Phenotypic and genotypic analyses of thymidine kinase-deficient isolates

Group	Strains	EC_{50} in Vero cells (μg/ml)[a]			Mutations in the TK gene[b]		Length of authentic TK sequence (aa)	Total length of polypeptide (aa)
		ACV	GCV	PCV	Nucleotide	Amino acid		
TK-positive	F	1.2 ± 0.4	0.5 ± 0.2	1.4 ± 0.1	None	None	376	376
	CL101[c]	Not tested	Not tested	Not tested	A427G C766T	I143V R256W	376	376
	AR1	44.4 ± 7.8	33.8 ± 4.5	>100	C246G T965A	No change M322K	376	376
TK-negative	AR3	30.6 ± 8.0	65.3 ± 15.0	98.2 ± 12.0	C533-	Q185R	184	407
	AR7	44.7 ± 6.5	66.6 ± 9.0	100.0 ± 14.0	G247T	E83K	376	376
	AR8	50.7 ± 11.0	26.8 ± 4.0	92.4 ± 20.0	G346A C460-	D116N P155R	154	181
TK-partial	AR2	57.5 ± 10.0	5.2 ± 2.0	9.5 ± 2.3	C566T	A189V	376	376
	AR4	31.5 ± 8.0	2.8 ± 0.9	9.5 ± 3.5	C566T T1043C	A189V V348A	376	376
	AR5	44.6 ± 12.0	17.9 ± 8.0	29.2 ± 6.0	C646T	R216C	376	376
	AR6[d]	>100	16.5 ± 3.1	7.0 ± 2.1	G488A	R163H	376	376
	AR9	50.0 ± 3.0	54.9 ± 6.0	14.3 ± 3.2	G488A T592C	R163H No change	376	376

[a] The concentration of a drug that is required for 50% cell survival of the cell cultures against the CPE of the virus strain at 3 days p.i. Data are the mean ± S.D. of a at least three independent experiments.

[b] 'C246G' represents the nucleotide substitution of cytosine (C) for guanosine (G) at position 246 of the ORF region of the TK gene. 'M322K' represents the amino acid substitution of Met for Lys at position 322.

[c] The complete nucleotide sequence of the thymidine kinase gene of HSV-1 strain CL101 determined by Wagner et al. (1981).

[d] Also mutation A719V in the DNA polymerase gene UL30.

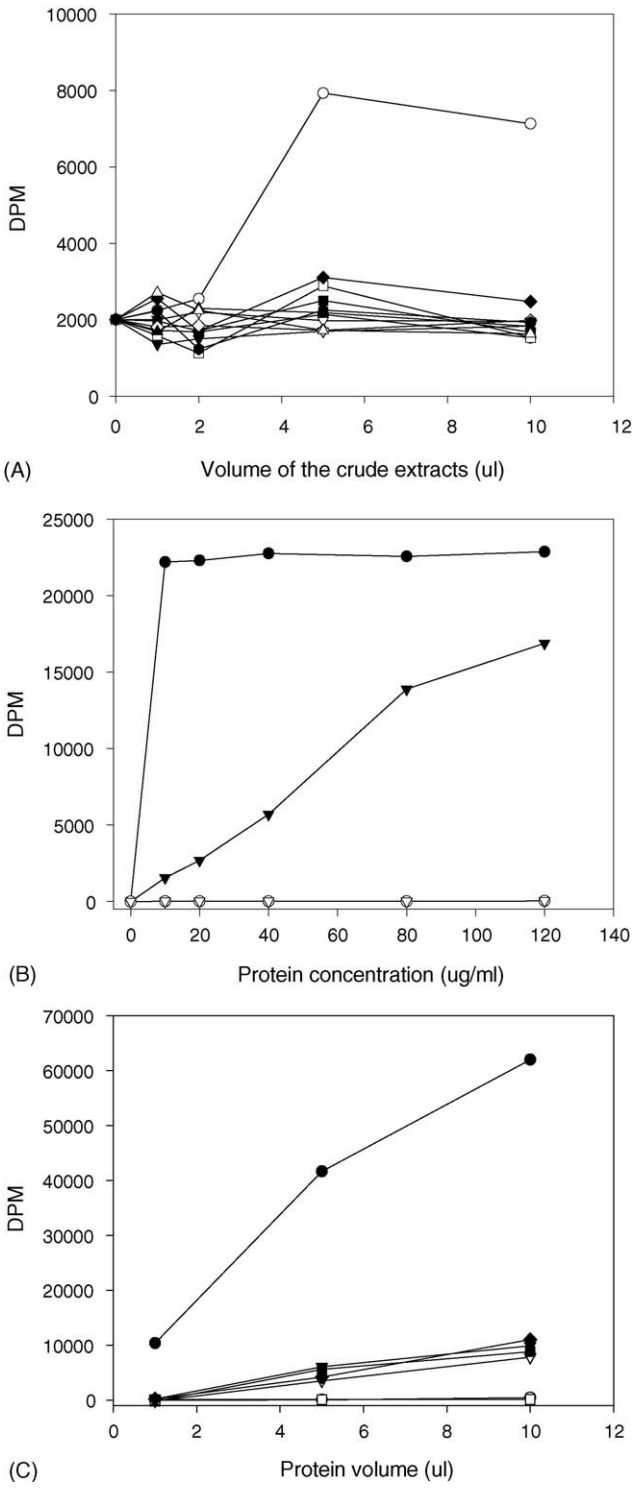

Fig. 1. Enzymatic activities of TK proteins: (A) vTK activity of virus-infected Vero cell lysates: mock (●), F (◯), AR1 (▼), AR2 (▽), AR3 (■), AR4 (□), AR5 (♦), AR6 (◊), AR7 (▲), AR8 (△), and AR9 (◆) strains. The volume 1 μl in this figure represents the lysate of 10^5 Vero cells; (B) vTK activity of GST-removed recTK proteins from *E. coli*: F (●), AR1 (◯), AR2 (▼), and AR7 (▽) strains; and (C) vTK activity of recTK proteins from *E. coli*: F (●), AR3 (◯), AR4 (▼), AR5 (▽), AR6 (■), AR8 (□), and AR9 (♦) strains. The concentration (μg/μl) of the recTK proteins were as follows: F, 0.49; AR3, 0.47; AR4, 0.22; AR5, 0.17; AR6, 0.05; AR8, 0.12; and AR9, 0.24.

in the TK gene of all the strains were reconfirmed by DNA sequencing the *E. coli* expression plasmids, pGEX-4T1, harboring the vTK gene of each mutant. The expression of the vTK gene in the virus-infected Vero cells was confirmed by western blot analysis probed with rabbit anti-TK polyclonal antiserum, in which bands with the expected sizes were observed (data not shown).

3.2. Intracellular uptake and incorporation of thymidine into DNA in Vero cells

The influence of the vTK on [^{3}H]Thd uptake was measured with virus-infected Vero cells under the one-step condition, as described in Section 2. The total radioactivity of the cell lysate containing [^{3}H]Thd was considered as the relative amount of Thd taken up. The count of the TCA-precipitates bound to the GF/C filterates was measured as the relative amount of Thd incorporated into the DNA. As shown in Fig. 3A, the Thd uptake in strain F increased to 6 h p.i. and remained at this level until the end of the replication cycle. The TK^p-mutants-AR2, AR4, AR5, AR6, and AR9-showed some Thd uptake but at levels which were lower than that of strain F and which varied from strain to strain. As in the case of the mock-infected cells, almost no Thd uptake was observed in any of the TK$^-$-mutants, AR1, AR3, AR7, and AR8.

Having shown that the vTK enhances the intracellular uptake of Thd, we measured incorporation of [^{3}H]Thd molecules into newly synthesized DNA, as described in Section 2 (Fig. 3B). Once again F showed substantial Thd incorporation, while all of the TK^p-mutants showed some level of incorporation, but less than that of F. There was little incorporation in the TK$^-$ mutant-and mock-infected cells.

3.3. Effect of acyclovir, ganciclovir and penciclovir on intracellular uptake and incorporation of thymidine into DNA

To determine the effect of exogenously added antiviral drugs on the Thd uptake and its incorporation into the DNA, these levels were measured in Vero cells infected with the mock, F, AR1, and AR2 in the presence of ACV, GCV or PCV. In the time-coursed experiments under the one-step condition, F showed increased [^{3}H]Thd uptake in the presence of ACV at all of the concentrations tested −0, 1, 2, 5, and 10 μg/ml (Fig. 4A). However, 1 μg/ml of ACV resulted in very low Thd incorporation into DNA and higher concentrations inhibited it completely (Fig. 4B). ACV at 10 μg/ml in TK^p AR2 inhibited neither the Thd uptake nor DNA replication completely, although the uptake levels were lower than those of F. No increased uptake caused by ACV was measured. The cells infected with the mock or TK$^-$ AR1 showed little Thd uptake or Thd incorporation. We also investigated the Thd uptake and its incorporation in the presence of GCV and PCV. [^{3}H] Thd was added to virus-infected Vero cells containing various concentrations (0, 1, 5, 10, 50, and 100 μg/ml) of GCV or PCV at 1 h p.i. The cells were harvested at 13 h p.i. for the analysis. As shown in Fig. 5, the order of the levels of Thd uptake was F > AR2 > AR1 and the mock-

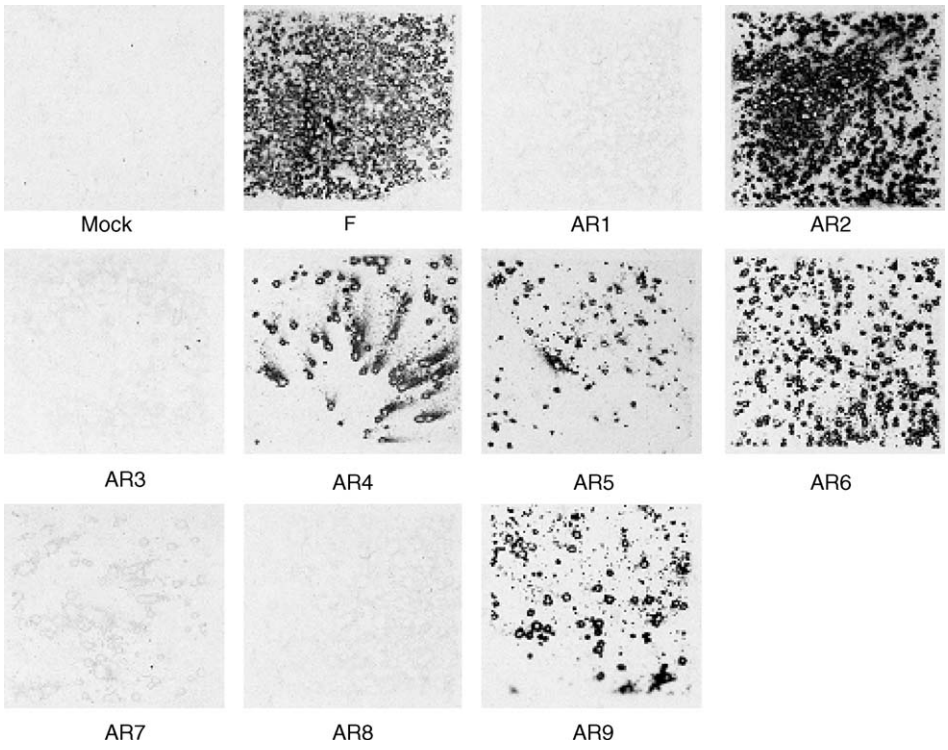

Fig. 2. Plaque autoradiography of virus-infected 143B cells. For cell culture a cover glass was dropped into each well of 6-well plates. The medium in the wells having a proper plaque number at 3 days p.i. was removed and the monolayer was incubated with 0.2 μCi of [^{14}C]Thd (specific activity 59.7 Ci/mmol) in 1.5 ml DMEM/2% FBS for 4 h at 37 °C. The cells were washed twice with PBS(−) for 15 min at 37 °C each time. The cells were then stained with crystal violet, washed, and air dried. The cover glasses were placed in contact with X-ray film for 5 days at −70 °C and developed in the dark room.

infected cells. At all of the concentrations of GCV and PCV tested, no notably increased uptake was measured, while that of ACV showed a 25–60% increase in the F-infected cells. As in the case of ACV, at those concentrations, which could inhibit the synthesis of the viral DNA, neither GCV nor PCV decreased the intracellular Thd uptake.

3.4. Influx and anabolism of acyclovir, ganciclovir and penciclovir in different cell lines

To study the influence of vTK on the influx and anabolism of vTK-dependent drugs, virus-infected cells were exposed to [^{3}H]-labeled ACV, GCV, or PCV for a period of 8 h, from 1 to 9 h p.i.-under the one step condition and harvested for analysis, as described in Section 2. Fig. 6 shows the amount of each compound and its anabolic products detected in each cell line. The total counts of the [^{3}H]-labeled compounds and their phosphorylated products were considered as the total influx. The influx level and amount of phosphorylated products of each compound declined generally in the order of F, AR2, AR1, and mock. In Table 2, the influx ratios of each compound are shown relatives to mock-infected cells. The difference in the total amount of PCV or GCV and their anabolic products in the F-infected Vero or 143B cells was significantly higher than that observed for the mock, AR1 or AR2-infected cells. The differences between AR1 and AR2 were more evident in GCV and PCV than in ACV. In the case of ACV, only a slightly increased influx was observed with F as compared to the mock, AR1 or AR2-infected cells.

The ratios of each precursor and its phosphorylated forms – monophosphate (MP), diphosphate (DP), and triphosphate (TP) – varied from compound to compound and sometimes from cell-line to cell-line. The metabolite patterns of each compound were generally less dependent on the vTK activity than the influx of each compound. In the F-infected 143B cells, the level of ACV-TP was the highest, followed by ACV-DP, ACV-MP, and ACV in that order. In the case of GCV, the majority of the molecules remained unmetabolized. In the 143B cells infected with other viruses, only a small amount of GCV was detected. The anabolism profile of PCV in the 143B cells were quite similar to that in the Vero cells, although the total influx of PCV was much higher than that in the Vero cells. The amount of anabolic products in the F-infected cells increased in the order: PCV-TP,-DP,-MP, and PCV.

4. Discussion

Various ACV-resistant laboratory isolates with different mutations in the TK gene were used for this study. We confirmed that the antiviral resistance of all of the mutants against ACV, GCV, and PCV was due to their TK-deficiency. They became sensitive to the antiviral agents in FTK143B cells (for AR1 and AR2 in Kim et al., 2002; other data not shown). We were able to classify them into two groups: vTK^{-} and vTK^{P} by measuring the enzyme activity of recTK proteins isolated from an *E. coli* expression system. We did not measure the difference in the activity among the vTK^{p} strains. However, unlike the

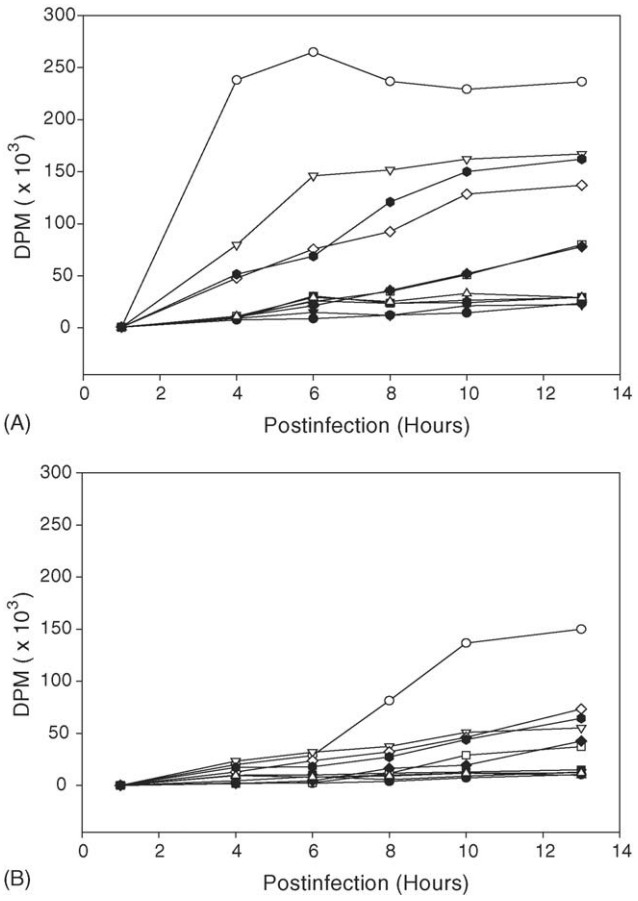

Fig. 3. (A) Intracellular uptake of [^{3}H]thymidine and (B) its incorporation into DNA in Vero cells infected with various strains of HSV-1 under the one step condition. HSV-1: mock (●), F (○), AR1 (▼), AR2 (▽), AR3 (■), AR4 (□), AR5 (◆), AR6 (◇), AR7 (▲), AR8 (△), and AR9 (⬢) strains. [^{3}H]Thd was added (0.5 μCi/well of 96-well plates) at 1 h p.i. and the cells were harvested in triplicate at various times thereafter. The final volume of the resuspension of the cells per well was 300 μl (17% trypsin-EDTA, 34% PBS(−), 10% TCA). Immediately after TCA-addition, the tubes were vortexed and 10 μl of the crude cell lysate was spotted on a Whatman paper for the uptake assay. After 30 min on ice, the TCA precipitates of the remaining lysate were collected on a GF/C filter for the DNA incorporation assay. The radioactivity that was obtained was recalculated for the original volume of each well, i.e. 300 μl. The average data of 3-wells is shown. All results are expressed as the arithmetic mean ± S.D. of three separate experiments. $^*P < 0.001$ compared with virus-infected cells.

vTK^p, which had some enzyme activity, the vTK$^-$ showed little activity. Enzyme assay with virus-infected cell lysates could not differentiate the vTK$^-$ from the vTK^p. A plaque autoradiogram using [^{14}C]Thd and cTK$^-$ 143B cells acted as a good tool to confirm the vTK$^-$ activity, although this method could not distinguish vTK^p from vTK$^+$ or cTK$^+$. It was also useful to monitor the homogeneity of the vTK$^-$ virus stock. Unlike the vTK^p group, which varied from strain to strain, the vTK$^-$ strains shared many common features in terms of the antiviral resistance, virulence and reactivation profiles in animal models (manuscript in preparation). For functional or mechanistic studies with vTK, it may be important to determine whether the TK-deficiency is vTK^p or vTK$^-$.

We have previously reported the M322K mutation in AR1 (Kim et al., 2002). AR7 had the E83K mutation (Suzutani et al., 2003). Based on X-ray crystallography studiess, it was suggested that E83 and R163 were important in the active site of TK:ADP:TMP/Thd in the catalytic reaction (Wild et al., 1997). Both the E83K and M322K mutations abolished the vTK activity completely, but R163H only partially. The vTK$^-$ activity of the frameshift mutation caused by the deletion or addition of nucleotide(s) has often been reported. Mutation A189V (with or without V348A) or R216C caused reduced enzyme activity. The mutation A719V of AR6 in the DNA polymerase gene was previously reported (Knopf, 1987; Larder et al., 1983), and this mutation together with the TK-mutation caused super-resistance to ACV (Kim et al., 2002).

The radioactivity of the cell lysates containing [^{3}H]Thd and its metabolites, either in the free form or bound to macromolecules, reflected the consequence of Thd uptake. The intracellular uptake of Thd in the virus-infected cells was not influenced by viral DNA replication. The intracellular uptake of Thd was highly dependent on the vTK activity of the viral strains. The vTK$^+$ strain F showed the highest uptake of Thd, the vTK^p strains showed moderate uptake, although the levels varied from one strain to another, and the vTK$^-$ strains showed little uptake. The dNTP pools in the mock-infected cells might be sufficient to support viral DNA synthesis. This idea was supported by the fact that the vTK$^+$, vTK^P, and vTK$^-$ viruses replicated to similar extents in cell line. The difference in the amounts of Thd uptaken among the viral strains might simply be a reflection of the accumulation of the phosphorylated products (i.e. the TMP produced by the vTK activity is subsequently changed to TDP and TTP).

However, the vTK activity may influence the Thd uptake, because, as shown in Fig. 3A, most of the vTK^p mutants having some vTK activity did not show a continuous increase in their Thd uptake. Only a slight decrease in the level of the Thd uptake in the F-infected cells was observed until the end of the one-step viral replication cycle. Using an immunofluorescence technique, we were able to detect the vTK proteins from 2 h p.i. to the end of the viral replication cycle leading to cell death. These proteins might be products of the continuous expression of the vTK gene, because its mRNA was detected continuously (manuscript in preparation). Preliminary data on the uptake of other deoxynucleosides (viz. deoxyadenosine and deoxygluanosine, and presumably dCyd) by Vero cells infected with the mock, F, AR1, or AR2 strain showed minimal differences among the different strains (data not shown). The very low levels of dCyd uptake made it difficult to determine with certainty, but no obvious increase in the level of dCyd uptake induced by F was observed. It is necessary to compare the anabolism of these nucleosides in cells infected with different viral strains.

ACV increased the Thd uptake, with or without viral DNA replication, in F-infected Vero cells but not in the AR1-or AR2-infected ones. GCV and PCV did not exhibit such a phenomenon. An opposite observation was reported in a previous study (Harmenberg et al., 1985), in which HSV-1 infection reduced the Thd concentration of African green monkey kidney cells and the inhibition of virus replication caused by ACV resulted at a Thd concentration similar to that observed in the mock-infected cells. It was reported that ACV stimulated the dCMP deaminase activity in HSV-1-infected cells, and

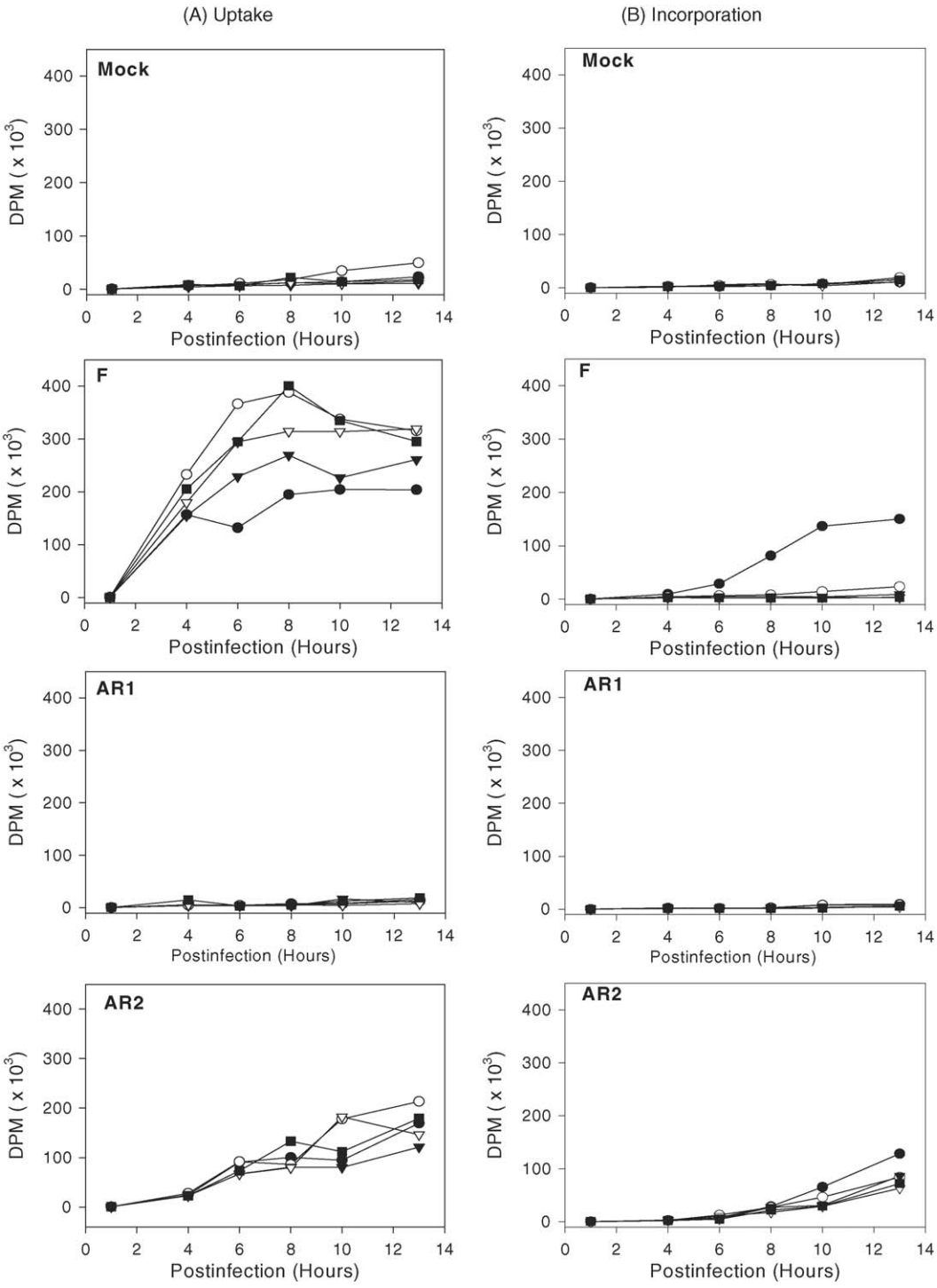

Fig. 4. Influence of acyclovir on: (A) intracellular uptake of [^{3}H]thymidine and (B) its incorporation into DNA in Vero cells infected with various virus strain under the one step condition. Acyclovir was added after 1 h of virus adsorption. The measurement was performed as described in Section 2. ACV: 0 μg/ml (●), 1 μg/ml (◯), 2 μg/ml (▼), 5 μg/ml (▽), and 10 μg/ml (■).

when DNA synthesis was inhibited completely by ACV, the de novo TMP synthesis was increased (Karlsson, 1991). In ACV^r strains, AR2 replicated even at the highest concentration of ACV tested, i.e. 10 μg/ml, but no evident influence of ACV on the increase in the uptake of Thd was found. Under this one step experiment, DNA replication of AR2 seemed to be affected by ACV at the lower concentrations tested (1–10 μg/ml) than the EC_{50} value of ACV measured by CPE inhibition at 3 days p.i. (57.5 ± 10.0 μg/ml). The inhibition of Thd incorporation into DNA did not decrease the Thd uptake at all, i.e. it occurred without viral DNA synthesis. The intracellular uptake of ACV, GCV, and PCV increased generally in the order of F, AR2, and AR1/mock. Differences between the cell lines, Vero and 143B, were also observed, especially in the case of GCV and PCV, but

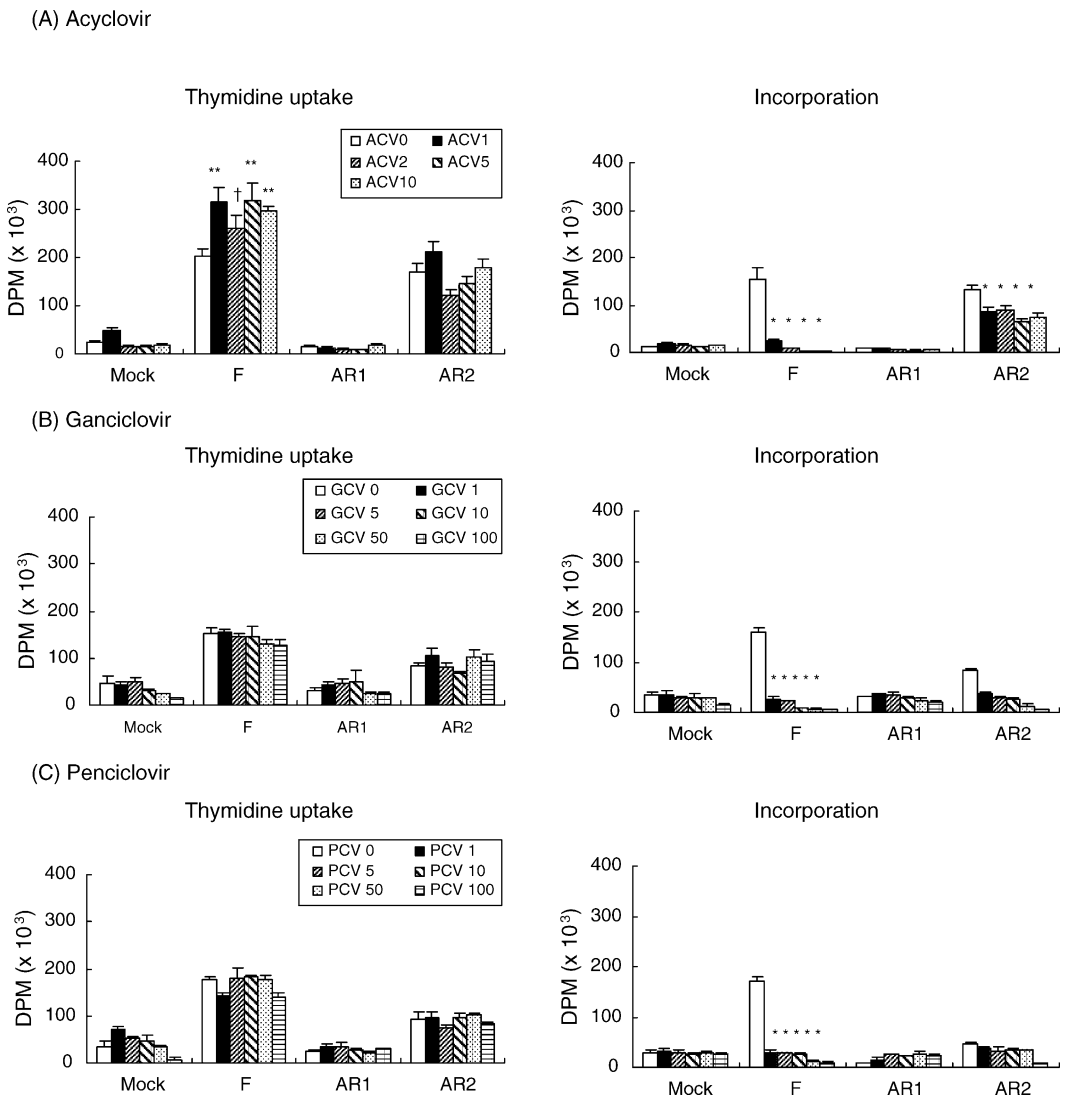

Fig. 5. Effects of: (A) acyclovir; (B) ganciclovir; and (C) penciclovir on intracellular uptake and incorporation of [^{3}H]thymidine into DNA in Vero cells infected with various virus strains and treated with various concentrations (μg/ml) of antiviral drugs under the one step condition. The compounds were added at 1 h p.i. and the cells were harvested at 13 h p.i. for the analysis. All results are expressed as the arithmetic mean ± S.D. of three separate experiments. $^{*}P<0.001$ compared with virus-infected cells that were not exposed to antiviral agent. $^{**}P<0.01$. $^{\dagger}P<0.05$.

Table 2
Influxes of thymidine, acyclovir, ganciclovir and penciclovir: influx relative to 143B cells and mock-infected cells

Compounds	Cell lines	Influx relative to 143B	Influx relative to mock-infected cells			
		Mock	Mock	F	AR1	AR2
Thymidine	Vero	4.1	1.0	52.2	1.2	3.2
	143B	1.0	1.0	116.0	1.7	3.7
	FTK143B	–	–	–	–	–
Acyclovir	Vero	2.7	1.0	2.6	1.0	0.7
	143B	1.0	1.0	9.1	1.6	2.1
	FTK143B	7.5	1.0	3.3	0.5	0.7
Ganciclovir	Vero	0.7	1.0	79.2	4.7	29.0
	143B	1.0	1.0	541.1	2.6	17.9
	FTK143B	201.8	1.0	3.3	0.8	1.5
Penciclovir	Vero	0.1	1.0	263.8	3.7	57.8
	143B	1.0	1.0	213.2	1.2	28.7
	FTK143B	98.2	1.0	1.8	0.9	1.4

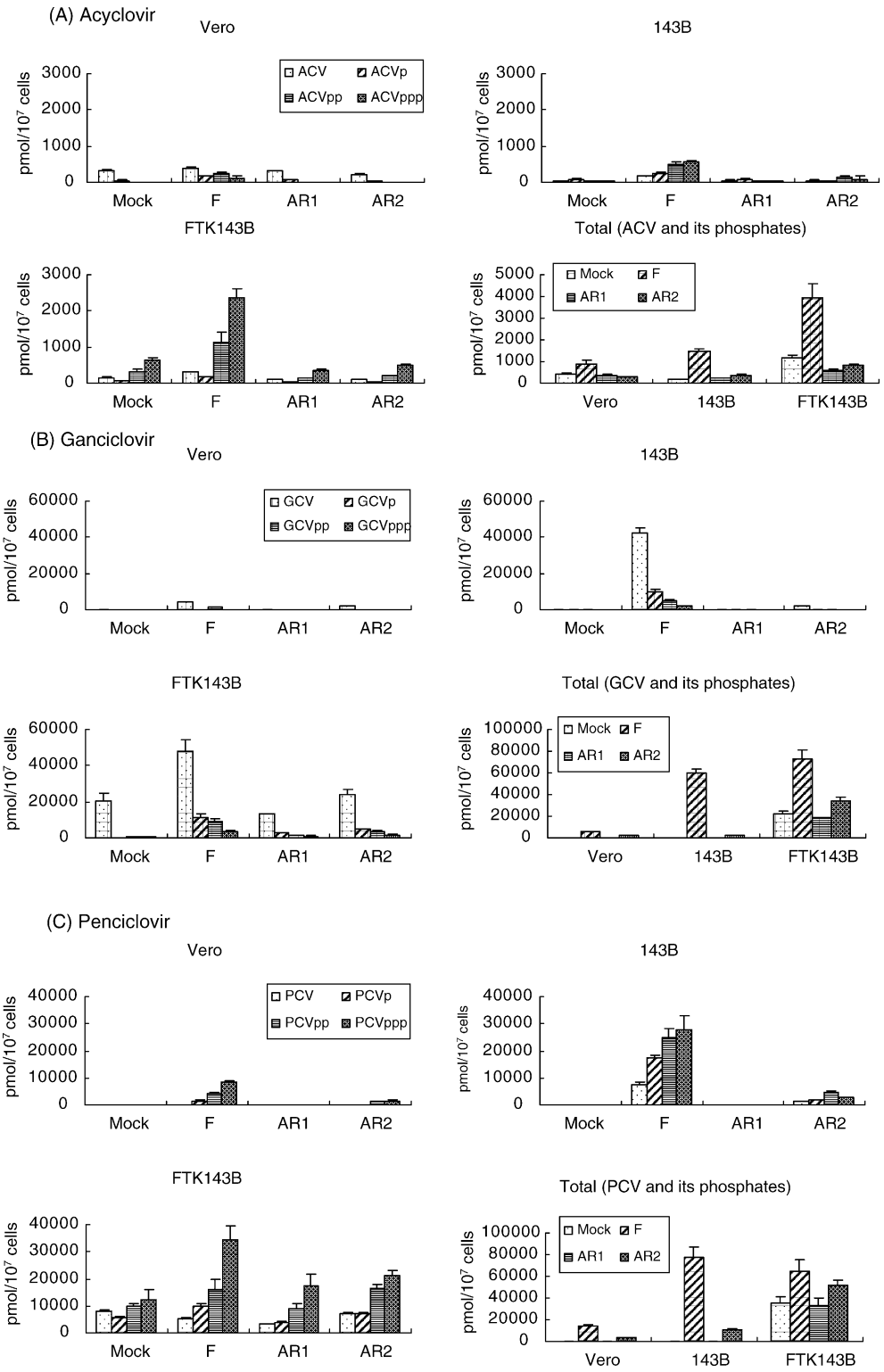

Fig. 6. Influx and anabolism of: (A) acyclovir; (B) ganciclovir; and (C) penciclovir in Vero, 143B and FTK143B cells infected with various virus strains under the one step condition. To the virus-infected cells in 100-mm Petri dishes, 1 μM [^{3}H]-labeled compound was added at 1 h p.i. To the cells harvested at 9 h p.i. 600 μl of cold 0.5 M perchloric acid was added and stored for 30 min at 4 °C. The supernatant separated by centrifugation was neutralized with 150 μl 4 M KOH and buffered with 1 M potassium phosphate buffer at pH 7.2. The supernatant obtained after centrifugation was evaporated under reduced pressure. The residue containing the nucleosides and nucleotides was dissolved in 1 M potassium phosphate buffer and analyzed. All results are expressed as the arithmetic mean ± S.D. of three separate experiments.

there was only a slight difference in the metabolites among the viral strains in each cell line.

The influence of the vTK was much less profound in ACV than in GCV or PCV. The FTK143B cells produced the vTK protein with functional activity, but the difference in the uptake levels of ACV, GCV, and PCV was minimal among the viral strains. The affinities of ACV, GCV, and PCV for purified HSV-1 TK were previously reported (Griffiths, 1995). These compounds were competitive inhibitors of Thd phosphorylation and could also act as alternative substrates, although the relative substrate efficiencies differ from each other. The guanosine analogs were not substrates for the cTK. Thd (K_m, 0.4 μM) (Larsson et al., 1986), GCV (K_m, 10 μM), and PCV (K_m, 1.5 μM) seemed to have a higher affinity for the vTK than did ACV (K_m, 100 μM). Both GCV and PCV were readily phosphorylated by the vTK than ACV, resulting in relatively high concentrations of the influxes of GCV and PCV in the vTK^+ cells.

The vTK is presumably necessary to accommodate the increased demand for thymidine triphosphate to fuel viral DNA synthesis, especially in resting cells such as neurons. In mammalian cells, Thd, uridine and other pyrimidine and purine nucleosides are apparently transported by facilitated diffusion (equilibrative nucleoside transport) and/or ion-nucleoside cotransport (concentrative nucleoside transport) with broad substrate specificity (Baldwin et al., 1999; Cabrita et al., 2002; Cass et al., 1999). Studies using inhibitors of nucleoside transport and also vTK inhibitors can provide more detailed information regarding the functions of vTK. The influence of vTK on cellular and viral replication and pathogenesis should also be further investigated.

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