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Incorporation of 5-Substituted Analogs of Deoxycytidine into DNA of Herpes Simplex Virus-Infected or -Transformed Cells Without Deamination to the Thymidine Analog

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The incorporation into DNA of 5-bromocytosine and 5-iodocytosine, derived from their respective administered deoxyribonucleoside analogs, has been demonstrated in studies with cells infected with herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and in cells transformed with the thymidine kinase gene of HSV-1. No significant incorporation of iodocytosine or iodouracil occurred in the DNA of uninfected or nontransformed cells when the deaminating enzymes were inhibited, in accord with past studies in our laboratory with 5-bromodeoxycytidine and tetrahydrouridine. When 2′-deoxytetrahydrouridine, a potent inhibitor of cytidine deaminase and dCMP deaminase, was utilized, all the counts in DNA that were derived from [^{125}I]iododeoxycytidine appeared as iodocytosine in HSV-infected cells. In the absence of a deaminase inhibitor, 32 to 45% of the counts associated with DNA pyrimidines appeared as iodocytosine, and 55 to 68% appeared as iodouracil in HSV-infected cells. Substantial incorporation of iodocytosine (16%) occurred in cells transformed with the HSV thymidine kinase gene, suggesting the importance of the specificity of cellular nucleoside kinases and the activity of the deaminases in presenting unmodified bases to an undiscriminating polymerase. Incorporation into DNA of bromocytosine derived from [^{3}H]bromodeoxycytidine was demonstrated in HSV-2 infected cells; very little incorporation of bromocytosine compared with bromouracil could be demonstrated in these cells in the absence of inhibition of the deaminases (19% of the total counts associated with pyrimidines with deaminase inhibition and 1.5% without). Limited studies with 5-methyl[5-^{3}H]deoxycytidine indicated essentially no (or very little) incorporation of this analog as such in the DNA of HSV-1- and HSV-2-infected and -transformed cells. This suggests an exclusion or repair mechanism preventing inappropriate methylcytosine incorporation in DNA. The addition of nucleoside and deoxyribonucleoside deaminase inhibitors, which leads to the incorporation of 5-halogenated analogs of deoxycytidine into DNA as such, does not impair their antiviral activity. We infer from studies with 4-*N*-alkyl (ethyl and isopropyl)-substituted analogs of iododeoxycytidine that they are incorporated as such into DNA without deamination and effectively inhibit the virus at concentrations that are marginally toxic. Among the several reasons presented for the heightened potential efficacy of analogs of deoxycytidine compared with those of deoxyuridine is that the former, as analogs of 5-methyldeoxycytidine, may impair viral replication by perturbing processes involving methylation and changes in the methylation of deoxycytidine in DNA which appear to be important for the process of HSV maturation. In addition, this capacity to perturb methylation may, in turn, be the key to their potential as agents affecting entry into or emergence from latency, a process in which dramatic changes in the postpolymer 5-methylation of deoxycytidine occur in the DNA of herpesviruses.

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In previous studies from this laboratory (10, 18, 20, 43), it was shown that the 5-halogenated analogs of deoxycytidine (dC) are more selective agents than the corresponding analogs of deoxyuridine (dU). De Clerq et al. (14) commented on

the fact that the 5-substituted 2′-dCs have attracted relatively little attention as potential antiviral agents and stated that they had confirmed the contention of Greer and his colleagues (10, 15, 16, 20, 24, 43) that 5-halogenated analogs of dC are indeed more selective inhibitors of herpes simplex virus (HSV) replication than are the analogs of dU.

Tetrahydrouridine (H_4U), an inhibitor of cytidine deaminase (4), was used in our past studies to prevent conversion at the nucleoside level in a strategy that exploited our findings that the 5-halogenated analogs of dC are poor substrates for mammalian dC kinase and thymidine kinase (TK) (11, 12) but are effective substrates for the pyrimidine nucleoside kinase of HSV (10, 15) and varicella-zoster virus (16, 23). Although cytomegalovirus does not possess a virus-encoded enzyme capable of phosphorylating 5-halo(dC) analogs (24), the dC analogs are nonetheless more effective than analogs of dU against this virus (24, 45). As nucleotides, the dC analogs are substrates for deoxycytidylate (dCMP) deaminase (which is only moderately inhibited by H_4U), resulting in the formation of the thymidylate analog. It has been inferred that viral inhibition results when this deaminated metabolite is further anabolized to the inhibitory triphosphate in infected cells.

We were interested in examining the possibility that 5-halogenated and 5-methylated analogs of dC might be incorporated as such into the DNA of HSV-infected and HSV-transformed cells. In the present study we utilized 2′-deoxytetrahydrouridine (2′-dH_4U), an inhibitor of both cytidine deaminase and dCMP deaminase that was first utilized by Maley and Maley (30). This intermediate-state analog must be anabolized by the target cell to 2′-dH_4UMP by a phosphotransferase (31) to inhibit dCMP deaminase effectively ($K_i = 10^{-8}$ to 2×10^{-8} M).

The use of 5-substituted analogs of dC makes it possible to determine (for cells transformed by the HSV TK gene) the roles of the host DNA polymerase on the one hand and the nucleoside and nucleotide kinases and converting enzymes on the other, in maintaining the base modification patterns in DNA. This is an important consideration in view of the degradation of DNA, as well as mRNA and heterogenous nuclear RNA containing 5-methylcytosine (40, 42).

Making the reasonable assumption that the 5-halo(dUXP) metabolites result in greater toxicity than 5-halo(dCXP) metabolites, the use of dH_4U offers greater selectivity with 5-halogenated analogs of dC that may be partially anabolized in uninfected cells by dC kinase, for dH_4U inhibits the formation of the 5-halo(dUMP) analog. For example, 5-bromo(dUTP) is a potent inhibitor of nucleoside diphosphate reductase (32). Heightened efficacy could be achieved only if the 5-halo(dCMP) analog retained a great portion of its antiviral activity when further anabolized to the cytidine deoxyribonucleoside triphosphate; it is this question of increased efficacy that the present study addresses. Another way to prevent deamination to achieve greater efficacy was explored in these studies by utilizing analogs with 4-amino substitutions that result in intrinsic resistance to deamination.

The studies of Summers and Summers (47), who described a simple assay for the presence of the HSV TK gene based on findings from our laboratory (10), have led to the general use of 5-iodo(dC) (IdC) with H_4U to detect and characterize the regulation of the HSV TK gene in studies involving recombinant DNA technology (35, 39a). In this assay, counts in DNA are determined which are derived from IdC and are not inhibited by H_4U. The assay does not measure incorporation of IdC into DNA and is not concerned with the nature of the base incorporated into DNA.

In the present studies, we demonstrate that IdC is incorporated as such into the DNA of HSV-infected cells as well as that of cells transformed with a restriction fragment containing the HSV TK gene. With dH_4U, all the counts derived from IdC in DNA appear as iodocytosine; without dH_4U, the counts appear in DNA as iodouracil and iodocytosine in HSV-infected cells. Bromo(dC) (BrdC) was found to be incorporated as such into the DNA of HSV type 2 (HSV-2)-infected cells. It is of interest that similar, though more limited, studies with 5-CH_3dC have resulted in no stable or detectable incorporation of this analog into the DNA of virus-infected cells and very little, if any, incorporation in transformed cells. These results may suggest exclusion by the nucleotide kinases or DNA polymerase or repair of inappropriate CH_3dC moieties in DNA. In view of the findings of Youssoufian et al. (49), who suggest that methylation of viral DNA may be involved in HSV latency, the incorporation of a 5-halogenated analog of dC as such into DNA may do more than lead to viral inactivation with diminished toxicity; because of perturbations of the postpolymer methylation of dC in DNA, it may also have desirable effects in preventing latency or emergence from latency in recurrent infections. When these analogs are incorporated into DNA, they may interfere with the process of synthesis of nonmethylated DNA, which appears to be an important part of virus maturation in productive infection, as the studies of Sharma and Biswal (44) have suggested.

(A preliminary account of these results was

presented at the 80th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 11 to 16 May 1980).

MATERIALS AND METHODS

Virus and cell culture. Sources and culture methods of virus and cell stocks and the methods of cytotoxicity and antiviral activity assays have been described previously (18). LH_7 cells, transformed with a restriction fragment containing the HSV type 1 (HSV-1) TK gene, were the generous gift of Richard Axel (Columbia University, New York). The line was maintained in Dulbecco modified Eagle medium plus 10% fetal calf serum and (per ml) 15 μg of hypoxanthine, 1 μg of aminopterin, and 5 μg of thymidine (HAT medium). Incorporation studies were performed in Dulbecco modified Eagle medium plus serum, and the LH_7 cells required one passage in HAT medium minus aminopterin (HT medium) before they would flourish in the incorporation medium.

Nucleoside analogs. 2'-dH_4U, 4-*N*-methyl-5-IdC (4*N*-MeIdC), 4-*N*-ethyl-5-IdC (4*N*-EtIdC), 4-*N*-isopropyl-5-IdC (4*N*-IpIdC), 5-IdC, and 5-BrdC were obtained by special arrangement from Calbiochem-Behring, La Jolla, Calif. 2'-Deoxytetrahydrouridylic acid was a gift from Frank Maley of the New York Department of Health, Albany, N.Y. H_4U was obtained via Leonard Kedda of the National Cancer Institute, Bethesda, Md.

Radiolabeled IdC. [^{125}I]IdC was the generous gift of William Summers (Yale University, New Haven, Conn.) prepared by direct iodination of dC (47). We estimate the original specific activity of our sample (approximately 10 mM) at 20 μCi/μmol. This compound rapidly decomposed chemically to an unknown substance bearing the radiolabel; this substance was not iodouracil, IdC, or iodocytosine and did not appear to be incorporated into trichloroacetic acid (TCA)-insoluble material.

[^{125}I]IdC incorporation studies of HEp-2 cells infected with HSV-1. HEp-2 cells were infected with HSV-1 or were mock infected at a multiplicity of infection of 3, and adsorption was allowed to proceed for 2 h. The 10-cm plates were then washed twice with phosphate-buffered saline (PBS) solution and were overlaid with medium with or without dH_4U (6 mM). At 5 h postinfection, a 2-h pulse of [^{125}I]IdC was begun (5 μCi per plate). After the pulse, the medium was removed, the plates were washed twice with cold PBS, and the cells were lysed with 1.5 ml of 0.5% Sarkosyl–0.5% sodium dodecyl sulfate for 30 min at room temperature. The lysate was scraped into reaction tubes and incubated with pronase (1 mg/ml) in PBS-EDTA at 37°C for 8 h. NaCl was then added to each tube to a concentration of 10% (wt/vol). Residual protein was extracted by shaking with an equal volume of chloroform-isoamyl alcohol (20:1), centrifuging, and retaining the upper aqueous layer. DNA was precipitated and washed with ice-cold 95% ethanol, pelleted, dried, and treated as described below.

Alkaline hydrolysis, RNase digestion, and DNase I digestion. The dried ethanol precipitation product was: (i) incubated overnight in 0.5 N NaOH at 37°C, reprecipitated with ice-cold 70% TCA, washed with 95% ethanol, and dried; (ii) incubated with 0.025 ml of RNase solution (2 mg/ml in 0.02 M sodium acetate–0.15 M NaCl, pH 5.5) plus 0.975 ml of PBS for 2 h at 37°C, reprecipitated with TCA, washed in ethanol, and dried; or (iii) incubated in a 1-ml reaction mixture containing 25 μg of DNase (2,812 U/mg; Worthington Diagnostics, Freehold, N.J., type D), 15% Triton X-100, 0.45 mM Na_2 EDTA, 1.9 mM $MnCl_2$, and 45 mM NaCl in Tris-hydrochloride buffer (pH 7.4) for 2 h at 37°C, reprecipitated with TCA, washed with ethanol, and dried.

Hydrolysis and chromatography of dried acid-insoluble material. Dried acid-insoluble material was hydrolyzed in 0.05 ml of 7.5 N perchloric acid (PCA) at 100°C for 1 h. Less than 0.5% deamination of iodocytosine occurs under these conditions of hydrolysis. The resulting solution was neutralized with 0.06 ml of 7.5 N NaOH, particulate matter was removed by pelleting, and 20 μl of the hydrolysate was spotted on Whatman 3MM paper. Iodouracil and iodocytosine were added as carrier bases, and resolution was achieved by descending chromatography with isopropanol-HCl-water (170:41:39) for 20 h (R_f) of iodouracil, 0.80; R_f of iodocytosine, 0.48). Radiation was assayed without scintillant in a Shearle descending-well gamma counter by counting for 2 min.

[^{125}I]IdC incorporation experiments with LH_7 cells. LH_7 cells (36) were grown for 48 h in media containing 7 μCi of [^{125}I]IdC per 10-cm plastic tissue culture dish. The washed monolayers from 10 plates were scraped, pooled, and hypotonically lysed by suspension for 12 h in 10 mM Tris-hydrochloride (pH 7.4)–0.005 mM RNase A–0.1% Nonidet P-40–10 mM NaCl. Nuclear and mitochondrial fractions were separated by sequential centrifugations at 500 × *g* and 10,000 × *g* after the addition of 20 ml of 0.6 M sucrose buffer in 25 mM Tris-hydrochloride (pH 7.4)–2 mM EDTA (34). Pellets were dissolved in 0.5% Sarkosyl–0.5% sodium dodecyl sulfate, DNA was hydrolyzed, bases were chromatographically separated, and radioactivity was determined as described above. Each chromatographic track was cut into 1-cm segments, and the gamma emission of each segment was assayed.

[^{3}H]BrdC incorporation studies with HSV-2-infected BHK cells. BHK monolayers were infected at a multiplicity of 3 and were incubated for 1 h with agitation. The inoculum was then washed off, and medium containing 10% fetal calf serum was added. After 1 h more (2 h postinfection), the monolayer was washed twice with PBS, and medium was added containing 2'-dH_4UMP. (dH_4U was not available when these experiments were undertaken, and [^{3}H]BrdC was not available when dH_4U was acquired). After 1 or 3 h of preincubation, [^{3}H]BrdC (New England Nuclear Corp., Boston, Mass.; generally labeled at 210 μCi/mol) was added for a 2-h pulse at 37°C. The experiment was terminated by washing the monolayers twice with cold PBS and adding 5 ml of cold TCA to each plate. The resulting precipitate was washed three times with 10 ml of 5% TCA and was rinsed twice under 95% ethanol on glass-fiber filters. After drying, the total radioactivity derived from each dish was determined. The experiment was done in triplicate. The addition of dH_4UMP did not affect the total counts in the TCA precipitate. Incubation of the TCA precipitate with 0.5 N NaOH for 18 h at 37°C did not change the total radioactivity. The TCA precipitate was hydrolyzed from the glass-fiber filters with

7.5 N PCA at 100°C for 1 h, neutralized, and spotted with carrier 5-bromouracil and 5-bromocytosine on Whatman 3MM chromatography paper with water-saturated butanol as solvent. PCA hydrolysis under these conditions results in less than 1% deamination of 5-bromocytosine (reference 10 and the present studies). The R_fs of 5-bromouracil and 5-bromocytosine are 0.4 and 0.6, respectively.

5-CH_3[5-^{3}H]dC incorporation studies. An 0.8-mCi portion of 5-CH_3[5-^{3}H]dC (custom synthesized by Amersham-Searle, Chicago, Ill.) was added to the plates 5 h postinfection; otherwise, the methods were identical to those described for IdC. PCA hydrolysis under these conditions results in less than 1% deamination of CH_3dC. PCA hydrolysis in the presence of 0.15 M NaCl does not affect the extent of deamination of a standard CH_3dC preparation. Nevertheless, in an extension of these studies, enzyme hydrolysis coupled with high-voltage electrophoresis (17) or high-pressure liquid chromatography (6) would be appropriate. When it was observed that essentially no methyl(dC) was incorporated into DNA after a pulse of exposure, the transformed or HSV-infected cells were grown for 2 to 3 days in the presence of labeled analog. For chromatographic analysis, 5 μl each of 30 mM thymine and 5-methylcytosine was added to 20 μl of the hydrolysate that was spotted on Whatman 3 MM paper. Water-saturated butanol (16 h) was used to separate thymine (R_f, 0.60) and 5-methylcytosine (R_f, 0.37). Counts were determined in a Beckman LS-100C liquid scintillation system. For these studies, an additional transformed cell line designated Ac4, which exhibits HSV-2 pyrimidine nucleoside kinase activity, was utilized (gift of F. Graham, McMaster University, Hamilton, Ontario, Canada [1]).

RESULTS

Cytotoxicity and antiviral studies. Representative data from a series of experiments in which the effect of H_4U and dH_4U on the cytotoxicity and antiviral (anti-HSV-2) activity of IdC and 4-*N*-alkyl IdC analogs was examined are shown in Table 1. It is important to note that dH_4U did not diminish the antiviral effect of IdC. This indicates that deamination at the nucleotide level is not necessary for inhibition of the virus. Cytotoxicity was reduced equally by the deaminase inhibitors. IdC is a poor substrate

TABLE 1. Cytotoxicity and antiviral activity of IdC and 4-*N*-alkyl amino analogs of IdC in the presence of H_4U or 2′-dH_4U

Deoxyribonucleoside	Concn (mM) of:			% Surviving HEp-2 cells[a]	Log_{10} PFU of surviving fraction[b]	
	Deoxyribonucleoside	H_4U	dH_4U		HSV-2[c]	HSV-1[d]
None				100 ± 4		
		0.4		100 ± 5	−0.0	−0.0
			0.4	100 ± 8	−0.4	−0.2
IdC	0.30			0.001	−2.3	−5.0
	0.30	0.4		87 ± 6	−2.0	−5.0
	0.30		0.4	84 ± 2	−2.3	−5.0
4*N*-MeIdC	0.075			83 ± 7	−2.0	
	0.075	0.4		88 ± 5	−0.9	
	0.75			83 ± 11	−2.4	
	0.75	0.4		87 ± 4	−1.8	
	7.5			8.0 ± 3	−3.5	−3.3
	7.5	0.4		86 ± 10	−2.3	−3.0
4*N*-EtIdC	5.0			82 ± 7	−1.2	−0.2
	5.0		2.0	81 ± 8	−1.8	−0.2
4*N*-IpIdC	5.0			83 ± 11	−2.2	−0.2
	5.0		2.0	79 ± 4	−2.8	−0.3

[a] HEp-2 cells were plated at a density of 5×10^5 cells per 60-mm dish. After 24 h, growth medium was removed and replaced with medium containing deoxyribonucleoside analogs. After 48 h of incubation, the cells were washed three times with PBS and dispersed with trypsin, and appropriate dilutions were replated in growth medium. After 7 to 9 days of incubation, the medium was removed, the plates were washed twice with PBS, and the colonies were stained with crystal violet. The percentage of survival was calculated as the average number of colonies (>50 to 100 cells) grown out from analog-treated cells divided by the number of colonies on untreated control plates.

[b] Confluent monolayers of HEp-2 cells were infected at a multiplicity of 0.05. After adsorption for 2 h at 37°C with agitation every 15 min, medium containing the analogs was added. After 48 h of incubation at 37°C, virus was harvested by three cycles of freezing and thawing followed by scraping and sonication. Virus titers were determined by plaque assay on BHK monolayers. Titers are expressed as log_{10} of the surviving fraction relative to untreated controls.

[c] Dose, 2×10^6 PFU/ml.

[d] Dose, 4.7×10^4 PFU/ml.

for mammalian dC kinase (12), with a K_m value of 1,000 μM compared with a K_m value of 2 μM for dC.

One may infer from cell culture studies that 4*N*-MeIdC is a moderate substrate for cytidine deaminase since the addition of H_4U decreased the cytotoxicity of the analog. The decrease in anti-HSV activity when H_4U was added indicates that although 4*N*-MeIdC is less toxic, it is also less potent in anti-HSV activity than its deaminated form, 5-iodo(dU). The studies shown here are representative of other studies with a range of concentrations of 4*N*-MeIdC which lead us to conclude that it is not more efficacious than IdC plus H_4U as an antiviral agent; nevertheless, there are practical advantages in using simplified protocols and avoiding the coadministration of a drug, even a nontoxic drug.

In view of the low toxicity of 4*N*-EtIdC and 4*N*-IpIdC, it is unlikely that they are deaminated at the nucleoside level. These analogs display their antiviral activity at very high concentrations, and 4*N*-EtIdC is only marginally inhibitory to HSV-2; nonetheless, they may serve as prototypes for more effective deaminase-resistant 5-substituted pyrimidine deoxyribonucleosides. Persistence of the antiviral activity in the presence of dH_4U indicates that as with IdC, deamination is not necessary for the antiviral effect of these analogs. Whereas 4*N*-MeIdC is also effective against HSV-1, the 4*N*-EtIdC and 4*N*-IpIdC analogs are ineffective against this virus.

The effects of H_4U and dH_4U on the cytotoxicity and anti-HSV-2 activity of IdC and the anti-HSV activity of 5-BrdC are shown in Table 2. The addition of H_4U results in a dramatic increase in cell survival, as we demonstrated in previous studies (20, 43). In the case of BrdC, a slightly lower survival was obtained with dH_4U than with H_4U. This effect may be due to the effect of dH_4U on lowering the levels of dUMP. The goal of heightened efficacy might be better served by a concentration range of both BrdC and dH_4U at which cell survival would exceed that obtained with the coadministration of H_4U.

Incorporation studies with [^{125}I]IdC in HSV-infected cells. Assuming that IdC and its analogs inactivate HSV by being incorporated into viral DNA, one of the following occurs: (i) these analogs are incorporated into DNA as such without prior deamination; (ii) a deaminase resistant to inhibition by dH_4UMP and capable of removing alkyl-amino groups is expressed in HSV-infected cells; or (iii) dH_4U is not anabolized by a phosphotransferase to dH_4UMP in HSV-infected cells. To gain direct evidence for the first possibility, we examined the iodinated pyrimidines derived from [^{125}I]IdC in the DNA of HSV-infected cells.

TABLE 2. Effect of H_4U and 2'-dH_4U on cytotoxicity and antiviral activity of 5-BrdC and 5-IdC

Deoxyribonucleoside	Concn (mM) of:		% Surviving HEp-2 cells[a]	Log_{10} PFU of surviving fraction[b]
	H_4U	dH_4U		
HSV-1 infection[c]				
None			100 ± 3	
	1.0		100 ± 5	−0.0
		1.0	94 ± 3	−0.4
BrdC (0.30 mM)			0.03	−5.0
	0.25		37 ± 6	−5.0
	1.0		89 ± 11	−5.0
		1.0	59 ± 3	−5.0
IdC (0.35 mM)			0.03	−5.0
	0.25		20	−5.0
	1.0		75 ± 2	−5.0
		1.0	68 ± 17	−5.0
HSV-2 infection[d]				
BrdC (0.30 mM)			0.09	−2.7
	0.25[e]		61 ± 1	−1.7
		0.25[e]	26 ± 5	−2.7

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.
[c] Dose, 2×10^6 PFU/ml.
[d] Dose, 4.7×10^4 PFU/ml.
[e] Administration of 0.25 mM H_4U or dH_4U alone had no effect on cell survival or virus inhibition.

An examination of the results in Table 3 indicates that IdC-derived pyrimidines were recovered only as iodouracil in the DNA of uninfected cells when deaminase activity was not inhibited. No counts above the background level were recovered from uninfected cells pulsed in the presence of dH_4U. Conversion of IdC to the thymidine analog was necessary for its incorporation into the DNA of uninfected cells. These results are consistent with the cytotoxicity data and previous studies from this laboratory (10–12, 15, 16). In HSV-1-infected cells, IdC was incorporated as 5-iodocytosine or 5-iodouracil in the absence of dH_4U, but only as 5-iodocytosine when deamination was prevented. To exclude the possibility that incorporation into RNA was responsible for the recovery of this TCA-precipitable material, alkaline hydrolysis (Table 3, experiment 2) and RNase and DNase hydrolysis (Table 3, experiment 3) were utilized to characterize the labeled material further. The reappearance of counts as iodouracil after treatment with alkali at 37°C is likely due to the instability of the 4-amino group under these conditions in view of the results obtained with RNase treatment in experiment 3. Treatment with RNase did not

TABLE 3. Analysis of counts derived from [^{125}I]IdC associated with 5-iodouracil and 5-iodocytosine in DNA of uninfected and HSV-1-infected cells and DNA of LH_7 cells transformed with the HSV-1 TK gene.

Cells supplying DNA	Treatment	cpm		% cpm as iodocytosine
		Iodouracil	Iodocytosine	
Expt 1[a]				
Mock-infected	None	600	0	0
Mock-infected + dH_4U	None	0	0	0
HSV-1-infected	None	2,400	1,900	44
HSV-1-infected + dH_4U	None	0	1,100	100
Expt 2				
HSV-1-infected	None	600	280	32
HSV-1-infected + dH_4U	None	0	330	100
	NAOH hydrolysis	180	380	68
Expt 3				
HSV-1-infected + dH_4U	None	10	95	91
	DNase	0	0	0
	RNase	10	85	89
Expt 4[b]				
HSV-1-TK-transformed:				
Nuclear fraction	None	1,900	360	16
Mitochondrial fraction	None	360	30	8

[a] Experiments 1 through 3: HEp-2 cells were infected at a multiplicity of 3; after 2 h, 6 mM dH_4U was added as indicated. After 3 h, a 2-h pulse of [^{125}I]IdC (5 μCi per plate) was initiated. The cells were then lysed and digested with pronase, and the preparation was deproteinized. DNA was then precipitated, washed in ethanol, dried, and treated as indicated. After treatment with nucleases or KOH, the DNA was reprecipitated with TCA, dried, and hydrolyzed in 7.5 N PCA at 100°C for 1 h and was subjected to chromatographic analysis.

[b] Experiment 4: LH_7 cells were grown for 48 h in medium containing 7 μCI [^{125}I]IdC per culture dish. Pooled washed monolayers from 10 plates were lysed, and nuclear and mitochondrial fractions were separated by differential centrifugation. Pellets were dissolved in Sarkosyl-sodium dodecyl sulfate, and the DNA was hydrolyzed for chromatographic analysis.

affect the extent and distribution of radiolabel, whereas treatment with DNase brought the counts down to background levels. The reduced counts in each succeeding experiment in Table 3 were due to the chemical decomposition of the [^{125}I]IdC, discussed above. HSV-2 was utilized in HEp-2 cells in a study similar to that summarized in Table 3. Without dH_4U, significant incorporation of undeaminated IdC occurred: 55% of the counts associated with nucleic acid pyrimidines was with iodouracil and 45% was with iodocytosine, essentially equal to the levels found with HSV-1-infected cells.

Incorporation studies with [^{125}I]IdC in cells transformed with the HSV TK gene. It is conceivable that the viral polymerase is essential for the incorporation of IdC into DNA. Alternatively, the mammalian DNA polymerase could also be capable of this incorporation, but the specificities of the nucleoside kinases limit the incorporation of IdC in uninfected cells, precluding a test of this hypothesis. We therefore employed the cell line LH_7 (37), in which a restriction fragment containing only the HSV-1 nucleoside kinase gene has been cloned. The original source of this gene was HSV-1 strain F. This is the same strain of virus that was utilized in the antiviral and incorporation studies. To eliminate the possibility that all the incorporation of IdC might be due to phosphorylation by mitochondrial TK and subsequent action of the mitochondrial DNA polymerase, which is known to readily incorporate thymidine analogs into DNA (15, 27), separate mitochondrial and nuclear fractions were prepared. dH_4U was not added in these studies since preliminary studies (with only one concentration of dH_4U and IdC) indicated that dH_4U blocks the incorporation of all IdC-derived counts into the DNA of this cell line. These results may be attributed to the elevated intracellular dCTP pools resulting from a decrease in the effector inhibition of nucleoside diphosphate reductase, which is due in turn to the decreased levels of TTP; this elevation of dCTP would, we propose, obliterate the incorporation of IdC into DNA. Diminished TTP levels in transformed cells result from inhibition of the formation of dUMP by dH_4U. Evidence for this speculation is currently being investigated in biochemical studies. In HSV-infected cells, the levels of dCTP are low, whereas the levels of TTP are extremely high (22); furthermore, the reductase encoded by the virus is unresponsive to changes in the level of TTP (38). This could explain the difference in results obtained with 6 mM dH_4U with HSV-infected and

-transformed cells. It is likely that lowering the concentration of dH_4U below the high level of 6 mM would lead to the incorporation of significant levels of IdC into the DNA of transformed cells. The higher counts in Table 3, experiment 4, reflect the use of a freshly prepared batch of [^{125}I]IdC. Iodocytosine was recovered from LH_7 DNA (Table 3), indicating that the viral polymerase was not essential for this event. Furthermore, the amount of label recovered as iodocytosine in the nuclear fraction equaled 16% of those counts, whereas it was only 8% of the counts recovered in the mitochondrial fraction. The higher percentage of incorporation as iodocytosine in the nuclear fraction eliminates the possibility that iodocytosine incorporation is due solely to mitochondrial DNA polymerase and suggests instead that the nuclear DNA polymerase incorporates this unique base into DNA when its anabolism to the nucleotide is not excluded by the substrate specificity of the kinase.

Labeled DNA recovered from nuclei of LH_7 cells was specifically digested to either 5′ or 3′ nucleotides, and each digest was then examined chromatographically with appropriate standards for hydrolysis to nucleosides either by 5′ or 3′ nucleotidase. The results of these studies indicate that IdC was associated, significantly, with a 3′ phosphate and therefore that IdC was incorporated internally in DNA in intranucleotide linkage.

Incorporation studies with [^{3}H]BrdC in HSV-2-infected cells. Table 4 shows the incorporation of counts derived from [^{3}H]BrdC associated with 5-bromouracil and 5-bromocytosine in the DNA of HSV-2-infected BHK cells. BrdC is seen to be incorporated as such, and the extent of incorporation is related to the length of time after the addition of the virus, the period of preincubation with 6mM dH_4UMP before the 2-h pulse of [^{3}H]BrdC, or both.

5-CH_3[5-^{3}H]dC incorporation studies. Studies with LH_7 and AC-4 cells indicate that no counts above background derived from 5-CH_3[5-^{3}H]dC were incorporated into TCA-insoluble material when dH_4U was coadministered. Details of experiments with these cell lines and with HEp-2 cells are shown in Table 5.

The apparent counts in DNA CH_3-cytosine are readily chased by formate. This is not the case with counts in DNA thymine, which are largely derived from the deamination of CH_3dC and CH_3dCMP. The variability of repeated determinations, the possible trailing by thymine, and the high variability of counts in the region of the paper above and below the methylcytosine spot make counts of 16 cpm (with Ac-4 cells) and 35 cpm (with LH_7 cells) above background insignificant. It should be noted, however, that when a similar experiment was done with uninfected HEp-2 cells, the addition of formate reduced the 5-methylcytosine counts to background levels (Table 5). Methionine was less effective than formate in reducing the counts associated with methylcytosine. This may be due to the fact that the reutilized label in the pyrimidines was not associated with the 5-methyl position only. Further experiments comparing uninfected cells with transformed cells would reveal the validity of the low apparent extents of incorporation in transformed cells. Appropriate determinations towards this end include the addition of unlabeled CH_3dC to the incubations (to determine whether the CH_3dC counts are chased less effectively than with formate) and the addition of nicotinamide, which inhibits the postpolymer methylation of DNA (44).

It is of interest that in HSV-infected cells, no counts above background associated with methylcytosine were detected with or without dH_4U (and without the addition of formate). This is

TABLE 4. Analysis of counts derived from [^{3}H]BrdC associated with 5-bromouracil and 5-bromocytosine in DNA of HSV-2-infected BHK cells[a]

Preincubation (h)	Inhibitor	Expt no.	cpm (above background)		% cpm as bromocytosine
			Bromouracil	Bromocytosine	
1	None		1,116	12	1.1
	dH_4UMP (6 mM)		1,026	47	4.6
3	None	1	861	14	1.6
		2	863	13	1.5
	dH_4UMP (6 mM)	1	737	140	19.0
		2	727	139	19.1

[a] At 2 h after cells were infected at a multiplicity of 3, dH_4UMP was added. After 1 or 3 h of preincubation with the deaminase inhibitor, [^{3}H]BrdC (210 μCi/μmol) was added for a 2-h pulse at 37°C, and the reaction was terminated by TCA precipitation. The precipitate was washed three times in TCA and twice in 95% ethanol and was subjected to acid hydrolysis, as described in Table 3, footnote *a*, for chromatographic analysis.

TABLE 5. Effect of dH_4U, formate, and methionine on ^3H counts in DNA thymine and 5-methylcytosine that are derived from 5-CH_3[5-^{3}H]dC[a]

Cells	Addition	Concn	cpm		
			Background	cpm above background	
				Thymine	Methylcytosine
LH_7 (HSV-1-TK- transformed)	dH_4U	2 mM	20	0	0
	None			2,677	546
	Na formate	1 μg/ml		1,977	35
AC-4 (HSV-2-TK- transformed)	dH_4U	2 mM	40	0	0
	None			286	305
	Na formate	1 μg/ml		318	16
HEp-2	dH_4U	2 mM	30	0	0
	None			225	107
	Na formate	0.1 μg/ml		256	24
		0.5 μg/ml		162	0
		1.0 μg/ml		216	2
	None		20	1,361	123
	Methionine	0.4 μg/ml		864	78
		4 μg/ml		725	67
		40 μg/ml		837	28

[a] Cells were grown for 48 to 72 h in medium containing 0.8 μCi of 5-CH_3[5-^{3}H]dC and the indicated additions. DNA was prepared for chromatographic analysis as described in Table 3, footnote *a*.

probably due to the fact that preformed nucleosides are utilized so extensively (the salvage pathway) in HSV-infected cells that very little formate is reutilized for pyrimidine ring biosynthesis. HSV-1- or HSV-2-infected cells that were grown for 3 days in 5-CH_3[5-^{3}H]dC had significant label in thymidine only. Addition of dH_4U did not diminish the counts in thymidine of ^3H derived from CH_3dC. Consistent with the suggestion that these counts in thymidine are not derived from postpolymer deamination of CH_3dC is the fact that the addition of unlabeled thymidine 2 h before the addition of [^{3}H]CH_3dC decreased the ^3H counts in DNA thymidine by 95 to 99%. Thus, our limited studies with 5-CH_3[5-^{3}H]dC suggest that the source of the ^3H label in the DNA of uninfected cells is entirely the result of deamination and subsequent catabolism of metabolites of CH_3dC followed by recycling through the formate pool. Heightened salvage in infected cells would preclude extensive recycling of the labeled methyl group. An additional reason for the differences obtained between infected and uninfected cells is that mature viral DNA does not contain 5-CH_3-cytosine.

DISCUSSION

The new principal of approaching heightened efficacy that emerges from these studies is to prevent deamination of the 5-substituted analogs of dC at both nucleoside and nucleotide levels. By adding 2′-dH_4U or introducing alkyl substitutions at the 4-*N* position it is possible to incorporate these analogs into DNA as such. This has been demonstrated directly with IdC and BrdC and inferred with two of the 4-*N*-alkyl-substituted analogs. Even without the coadministration of an inhibitor of deamination, incorporation of iodocytosine was seen in the DNA of cells possessing high levels of cytidine and dCMP deaminase. This may be due, in part, to the fact that HSV TK is also a TMP kinase (5); therefore, the nucleotide may proceed to the diphosphate without leaving the enzyme, so that it is not accessible to dCMP deaminase.

A demonstration of the principal of achieving heightened efficacy by preventing deamination of the 5-substituted analogs of dC at both nucleoside and nucleotide levels was provided by experiments in which intrinsically deaminase-resistant IdC analogs were shown to inactivate HSV-2 significantly with only marginal toxicity in cell culture. The fact that these analogs continue to display their antiviral effect in the presence of dH_4U leads us to believe that the 4-*N* analogs can be incorporated as such. These results suggest further insight into the broad specificity of the HSV nucleoside kinase on which we first reported several years ago (10). Not only will 4-NH_2-5-halogenated pyrimidine analogs act as substrates for this enzyme, but extensive substitutions may be tolerated in the 4-*N* position. Because a number of substitutions at the 4-*N* position effectively prevent deamination of dC (28), other 4-*N* substitutions should be examined; furthermore, substitutions at the 4 position itself may be fruitful. The coadministra-

tion of dH_4U with IdC did not reduce the anti-HSV activity of these analogs, suggesting that nucleotide-level deamination is not necessary for virus inactivation as previously thought (10, 20, 43). Consistent with these observations are the results of incorporation studies with [^{125}I]IdC, which provide direct evidence for the incorporation of iodocytosine residues in DNA. Counts derived from [^{125}I]IdC were recovered only as iodouracil in uninfected HEp-2 cells, and no incorporation above background was detected in uninfected cells when deamination was inhibited by dH_4U. This agrees with the protection from cytotoxicity that is provided by dH_4U coadministration. In view of our findings that dH_4U resulted in no detectable counts associated with iodouracil in DNA, we may conclude that the presence of iodocytosine in DNA results in antiviral activity. Although the incorporation experiments were done under different conditions from those of the virus inhibition studies, the former were pulse exposures and therefore may underestimate the extent of incorporation of IdC or BrdC that occurs upon 48 h of exposure of the analog in virus inhibition studies. On the other hand, if a delayed repair mechanism is involved in removing the analogs from DNA, then the pulse exposures reflect higher levels of incorporation than those occurring in the virus inhibition studies. Virus inhibition studies with lower concentrations of IdC and BrdC with and without dH_4U, as well as incorporation studies more closely following the conditions of virus inhibition studies, are necessary before it can be stated unequivocally that incorporation of 5-halocytosine in DNA is another mechanism of virus inhibition. Because thymidine, but not dC, was found to protect HSV against inactivation by IdC, Renis (who did not coadminister inhibitors of deamination) argued against the possibility that IdC itself is responsible for the antiviral activity (39). Thymidine antagonism of IdC inhibition of HSV may not reflect a competition for incorporation but rather a competition for anabolism to the triphosphate. As we have shown (15), dC is a much poorer substrate for the HSV nucleoside kinase than IdC, BrdC, or thymidine, and therefore, competition at the kinase level could account for the observations of Renis. It is for this very reason that we contend that dC will be an excellent metabolite to coadminister at moderate concentrations to selectively antagonize toxicity when 5-halo(dC) and dH_4U are administered as an antiviral combination. Although the addition of dC to IdC or BrdC in the presence of dH_4U was not pursued in the current studies, our past studies indicate that this will be a fruitful approach in vivo. Antagonism of cytotoxicity was demonstrated in cell culture studies in our laboratory with 5-chloro(dC), dH_4U, and dC (18).

The studies of Sharma and Biswal (44) indicate that methylation of early synthesized DNA and demethylation (more likely, nonmethylation) of DNA late in the virus replication cycle may be an important part of the HSV maturation process. Incorporation of 5-halo(dC) analogs as such could perturb these processes; for example, methylation could misguidedly continue throughout the viral replication period. In addition, IdC may readily undergo tautomeric shifts and modify base stacking to present problems in DNA replication and transcription and in virus packaging; this could happen in a manner related to the molecular basis for the inactivation of viruses by IdU. IdU could affect methylation only very rarely, when the base in DNA undergoes a tautomeric shift. Although this is a momentary change, it could have long-term and significant effects in view of the facts that methylation of DNA is a processive event and that changes at one site could affect the methylation of an entire molecule downstream. Naturally, unless mispairing plays an important role in the disruption, the 5-halo(dC) analog could be assumed to have more profound effects on the methylation pattern in DNA.

The counts associated with iodocytosine obtained with HSV-infected cells were presumed to exist in viral DNA. Indeed, the extraction procedures used would favor recovery of viral rather than host DNA (37). The recovery of iodocytosine-associated counts from nuclear DNA of LH_7 (HSV-1 TK-transformed) cells grown in the presence of [^{125}I]IdC indicates that only the failure of 5-substituted dC nucleotides to form or their rapid deamination prevents their utilization by the cellular DNA polymerases in (nontransformed) mammalian cells.

The incorporation of IdC as such into DNA suggests that 5-methyl(dC) might be similarly incorporated. DNA methylation appears to regulate a variety of cellular functions (3, 7, 41), and studies with 5-azacytidine used to alter methylation patterns and affect transcription and differentiated functions are consistent with this concept (8, 9, 25). In view of the substantial 5-methylcytosine content in DNA (2 to 6% of cytosine residues are methylated [7]) and the degradation of DNA that occurs in many cells, it is conceivable that 5-methyl(dC) and 5-methyldeoxycytidylate could be reutilized for DNA synthesis with devastating effects on cellular control and gene expression were it not for the specificity of the mammalian nucleoside kinase and the activities of cytidine and dCMP deaminase coupled with the high K_m (0.6 mM) of dCMP kinase for its substrate, dCMP (the latter value was reported by Jackson [21]). dCMP deaminase may play a critical role, not only by providing a source of dUMP for thymidylate synthetase (for which there is an alternate de

novo pathway), but by preventing methyl(dCMP), which arises from the degradation of DNA, from being incorporated into DNA. The strategic location of ribonucleotide reductase at the diphosphate level rather than the monophosphate level may have resulted from the need (in the evolutionary sense) to expose the rare modified ribonucleoside monophosphates, which are derived from the processing of heterogenous nuclear RNA and the degradation of mRNA, to the discriminating substrate specificities of nucleotide kinases to prevent their incorporation into the genetic material at sites not specified by the enzymes of postpolymer methylation. In this regard, it would be of interest to determine whether 5-CH_3CDP would serve as a substrate for the reductase and to determine how efficiently 5-CH_3CMP is phosphorylated. In the studies of Creasey (13), 5-methylcytidine and 5-methyl(dC) possessed the lowest K_m value (lower than cytidine or dC) with respect to cytidine deaminase of 15 cytidine analogs examined. Similarly, Maley (29) has shown that 5-methyldeoxycytidylate has the lowest K_m value with respect to dCMP deaminase of chick embryo and monkey kidney cells among several analogs examined. Like cytidine deaminase, 5-methyl(dCMP) had a lower K_m value than dCMP. Perhaps this is to compensate for the fact that the K_m value of CH_3dC with respect to mammalian dC kinase is precariously low, as our studies showed (K_m of dC = 2 μM and K_i of CH_3dC = 18 μM for calf thymus cells; K_m of dC = 11 μM and K_m of CH_3dC = 71 μM for mouse P388 cells [11]); according to a study from another laboratory, an incredible value of 4 μM was obtained for CH_3dC, compared with a value of 2 μM for dC, with purified enzyme from calf thymus (26). It appears from the studies of Sköld (46) that 5-methylcytidine would not be a substrate for uridine kinase or cytidine kinase since 5-methyluracil is not a substrate; however, 5-methylcytidine itself was not examined.

It is of interest that we could not demonstrate substantial or even significant incorporation of 5-CH_3cytosine derived from 5-CH_3[5-^{3}H]dC from TCA-precipitable material from a cell line transformed with HSV-1 TK (LH_7), a cell line transformed by HSV-2 (Ac-4), HEp-2 cells infected with HSV-1, or uninfected HEp-2 cells. If any incorporation did occur, it was close to the limitation of the resolution of methods that were adequate for demonstrating iodocytosine incorporation and the presence of labeled 5-CH_3dC in DNA that was due to reutilization of the labeled methyl groups for the postpolymer methylation of cytosine and de novo pyrimidine biosynthesis. Further studies with 5-CH_3dC labeled at positions other than the 5 position (which we have had synthesized) are planned so that we can determine whether postpolymer demethylation or deamination occurs or whether some repair processes or degradation mechanisms are active in removing inappropriate 5-CH_3cytosines from DNA. Exclusion could be at the level of nucleotide kinases and DNA polymerase; although CH_3dCTP serves as a substrate for polymerase I in vitro (2), it may not in vivo. An unexpected exclusion occurs with RNA polymerase in the case of 5-fluoroCTP (19). Another exclusion mechanism for which there is a precedent in bacteriophage-infected cells involves a dCTPase that is highly specific; in this case, it does not affect hydroxymethyl(dCTP) but does hydrolyze dCTP. These aspects of CH_3dC involve considerations that go beyond the domain of viral chemotherapy and relate to current problems in molecular biology regarding regulation and differentiation.

The use of 5-halogenated analogs of dC, which have been shown to be potent antiviral agents (14; studies from this laboratory [10, 16, 18, 23, 24, 43]), may allow heightened efficacy in the management of productive infections. By coadministering inhibitors of their deamination or modifying the amino group so that deamination does not occur, the analog (i) will be a poor substrate for the mammalian nucleoside kinases but will be specifically phosphorylated by the HSV-encoded nucleoside kinase, unlike some dU analogs; (ii) will not be catabolized by uridine and thymidine phosphorylase, as are some analogs of dU; (iii) will not inhibit the host nucleoside diphosphate reductase as the triphosphate, as with E-5-propenyl(dUTP), an effective inhibitor of the host reductase (33) (counter to the goals of chemotherapy, these dU triphosphates may only weakly inhibit the effector-resistant nucleoside diphosphate reductase of HSV-infected cells); (iv) will not be dehalogenated at the nucleotide level by thymidylate synthetase, as is (for example) IdUMP (48); (v) will not inhibit thymidylate synthetase, an enzyme important for host but not for viral functions (a special situation for 5-nitro and 5-trifluoromethyl substitutions, for example); (vi) will allow the addition of dC, a selective antagonist that will allow heightened efficacy because it does not diminish the antiviral effect of these dC analogs, whereas it does diminish toxicity; (vii) will affect the methylation of DNA, which appears to be an important part of the maturation process of herpesviruses.

In view of recent experiments by Youssoufian et al. (49), who found that cytosine in the DNA of herpesviruses is methylated at the 5-position in a model system that is analogous to the latent state and unmethylated during productive infection, the selective incorporation of 5-halogenated analogs of dC into DNA as such could affect entry into and emergence from the latent state. The approach with 5-halogenated analogs of dC

might be fruitful even if the state of methylation does not determine whether the course of latency or productive infection is followed but is merely a distinguishing characteristic of two different states of the virus.

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