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Cell-specific antiviral activity of 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) against Marek's disease herpesvirus and turkey herpesvirus

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Summary

Three new fluoroarabinosylpyrimidine nucleosides (FIAC, FIAU and FMAU) were tested for in vitro activity against oncogenic and nononcogenic strains of Marek's disease virus (MDV) and herpesvirus of turkeys (HVT). Marek's disease is a herpesvirus-induced lymphoma in chickens. Nononcogenic strains of MDV and HVT can protect against this disease. All viruses were inhibited by 1 μM of these drugs in chick kidney cell (CKC) cultures, but only FMAU and FIAU were active in chicken embryo fibroblast (CEF) and spleen cell cultures. It was determined that whereas CKC produced the enzyme 2'-deoxycytidine-deaminase which is needed to deaminate FIAC to FIAU, CEF were devoid of this enzyme activity. In addition, the deaminase inhibitor 3,4,5,6-tetrahydrouridine prevented the antiviral activity of FIAC in CKC. FMAU was not active against two Marek's disease-derived lymphoblastoid tumor cell lines.

Marek's disease virus; herpesvirus of turkeys; anti-herpesvirus drugs; FMAU; FIAC; FIAU

Abbreviations: CEF, chicken embryo fibroblasts; CKC, chicken kidney cell; dCyt-deaminase, 2'-deoxycytidine-deaminase; FFU, focus-forming unit; FIAC, 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine; FIAU, 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodouracil; FMAU, 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil; HSV-1, herpes simplex virus type 1; HVT, herpesvirus of turkeys; MD, Marek's disease; MDV, Marek's disease virus; THU, 3,4,5,6-tetrahydrouridine; TK, thymidine kinase.

Introduction

The search for selective antiviral drugs has resulted in the development of a number of compounds with activity against several groups of viruses, especially herpesviruses [28]. Recently, several new compounds with promising antiherpesvirus activity, 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodocytosine (FIAC), 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodouracil (FIAU) and its thymine analogue (FMAU) were synthesized [31]. These three fluoronucleosides have been shown to have potent anti-herpes simplex virus activity in cell culture and in animal models [13,14,21,27,30]. Moreover, FIAC has been reported to inhibit cell lines derived from Burkitt lymphomas, some of which are positive for Epstein–Barr virus [8]. It was, therefore, of interest to test these antivirals against Marek's disease herpesvirus (MDV), a virus that causes lymphomas of thymus-derived lymphocytes in chickens and against two closely related but nononcogenic herpesviruses, the serotype 2 SB-1 strain of MDV and the FC126 strain of turkey herpesvirus (HVT). Both HVT and SB-1 are currently used commercially to vaccinate chickens against MD. All 3 viruses are highly cell-associated in vitro and in vivo (reviewed by Calnek [3]).

In this study, we determined that, whereas FIAC, FIAU and FMAU were potent inhibitors of MDV and HVT in chick kidney cells (CKC), only FMAU and FIAU were active against these viruses in chick embryo fibroblasts (CEF) and splenic lymphocyte cultures. However, FMAU had no inhibiting effect on continuously growing MD lymphoblastoid cell lines. Evidence is presented which suggests that the cell-dependent antiviral activity of FIAC is due to the lack of 2′-deoxycytidine (dCyt)-deaminase activity in uninfected and MDV- or HVT-infected CEF. This enzyme is responsible for the deamination of FIAC to FIAU, an important antiviral metabolite of FIAC [10,18,23].

Materials and Methods

Antiviral compounds

FMAU, FIAC, and FIAU were kindly provided by Dr. J.J. Fox and K.A. Watanabe (Sloan-Kettering Institute for Cancer Research, Rye, NY). Stock solutions of the drugs were dissolved in double-distilled water at a concentration of 1 mM, sterilized through 0.45 μM Millex-HA filters (Millipore Corporation, Bedford, MA) and stored at −70°C until use. In addition, 3,4,5,6-tetrahydrouridine (THU) was a gift from Dr. T.C. Chou (Sloan-Kettering Institute for Cancer Research, New York, NY). This compound is a potent inhibitor of dCyt-deaminase. Stock solutions were prepared as described above at a concentration of 10 mM.

Cell cultures and media

Primary CKC cultures were prepared from 2–3-week-old specific pathogen free chicks as previously described [4]. CEF were used as secondary cultures. Both CKC and CEF cultures were cultivated at 39°C in a medium consisting of M199 (GIBCO Laboratories, Grand Island, NY), 10% tryptose phosphate broth, 0.73% sodium

bicarbonate (10% w/v) and antibiotics. Bovine fetal serum was used at 5% and 2% for growth medium of CKC and CEF, respectively. After infection, CKC cultures were maintained under agar medium while CEF were maintained in liquid medium containing 0.25% bovine fetal serum.

Splenic lymphocytes for short term cultures were prepared from 5–8-week-old specific pathogen free chickens. Spleens were collected aseptically and gently forced through a 60 μm autoclavable screen (Tetco, Inc., Elmsford, NY) and centrifuged over a Ficoll–Hypaque (Pharmacia, Piscataway, NJ) gradient for 15 min at 800 × *g*. Cells were resuspended in LMH medium at a concentration of 4×10^6 cells/ml and incubated at 41°C in 5% CO_2 for 48 h. LMH medium consisted of equal parts Leibovitz L-15 and McCoy 5A media (GIBCO Laboratories, Grand Island, NY) supplemented with 5% tryptose phosphate broth, 8% bovine fetal serum, 10% chicken serum, 2 mM glutamine, 1 mM sodium pyruvate and 10 μM 2-mercaptoethanol and antibiotics [6,7].

Two MD lymphoblastoid cell lines, MDCC-CU12 and MDCC-CU36, were maintained under conditions identical to those used for the short term spleen cell cultures. Cell passages were made at 48-h intervals. MDCC-CU12 has a low level of expression of viral antigens while MDCC-CU36 has a high level of expression [6].

HEp-2 and Vero cells, free of mycoplasma, were obtained from Flow Laboratories (McLean, VA) and maintained as previously described [27]. Secondary rabbit kidney cells were prepared as previously described [9]. HeLa (Bu 25) cells were obtained from Dr. Y.C. Cheng (University of North Carolina, Chapel Hill, NC) and maintained as previously described [9].

Virus strains and assays

All MD virus strains were cell-associated and from stocks which were stored at −196°C. The nononcogenic SB-1 strain of MDV [25] was the 11th passage in CEF. The HVT-4 clone of FC-126 virus [5] was also grown in CEF and was passaged 32 times. The oncogenic MDV clone GA-5 [2] and JM-16 (Schat, unpublished data) were passaged 22 and 9 times, respectively, in CKC. The oncogenic MDV clone JM-10 was used in its 71st passage in spleen lymphocytes as described by Calnek et al. [7]. Virus assays were performed by the detection of characteristic foci at 7 days post infection (p.i.) for GA-5 in CKC, 5 days p.i. for SB-1 in CEF and CKC and at 3 days p.i. for HVT in CEF. Virus infection in splenic lymphocytes and expression of viral antigen in the MD cell lines were detected by staining acetone-fixed lymphocyte smears with fluorescein isothiocyanate-conjugated chicken anti-MDV serum [7]. Herpes simplex virus type 1 (HSV-1) strain F was obtained from Dr. B. Roizman (University of Chicago, IL) and a high titered pool was prepared as described previously [26].

Antiviral assays

FIAC, FIAU or FMAU were added to the cultures just prior to the inoculation of 100–500 focus-forming units (FFU) of virus per culture. The following concentrations of drugs, prepared in maintenance medium, were used: 1.0, 0.5, 0.1, 0.01 and 0.001 μM. Media were changed at 3 days p.i. for CEF, while CKC received an agar overlay at 1 day p.i. and were fed at 3 and 5 days p.i. The replacement media contained drugs in the

same concentrations as were present before. In one experiment, GA-5-infected CKC cultures were not exposed to FMAU or FIAC until 24 h after infection. Numbers of FFU in treated cultures were compared with those in untreated controls and a percentage determined to express the degree of antiviral activity. In all experiments, cultures were exposed to FMAU, FIAU or FIAC alone to monitor toxicity. In one experiment, THU was used at a concentration of 400 μM to test the effect of this deaminase inhibitor on the antiviral activity of FIAC and FIAU.

Antitumor assays

MD lymphoblastoid cell lines were cultured at a density of 5×10^5 cells/ml in the absence or presence of 1 μM FMAU. Cell counts were made at 48 h intervals and cells were reseeded at 5×10^5 cells/ml in fresh medium. Cell smears were made at each interval and the number of antigen-positive cells was determined. The possible development of viral resistance to FMAU was investigated after a total of 24 culture days by virus isolation in CKC with or without 1 μM FMAU in the medium.

Enzyme activity

Various virus-infected and uninfected cell cultures were assayed for thymidine kinase (TK) activity, or dCyt-deaminase activity, or both. Uninfected cells and virus-infected cells were harvested at 4 days post-seeding as follows. The medium was removed and the cells were washed with phosphate-buffered saline (pH 7.4). The cells were then harvested in 25 mM Tris-HCl (pH 8.0) by agitation with glass beads. The cells were pelleted at 4°C by centrifugation for 10 min at 200 × *g* and stored at −70°C until assayed.

For TK activity assays, the cell pellets were resuspended in 3 volumes of cold extraction buffer (10 mM Tris-HCl, pH 7.5, 3 mM dithiothreitol, 1.5 mM $MgCl_2$, and 50 μM thymidine). The cell suspensions were frozen and thawed 4 times and the salt concentration was adjusted to 150 mM KCl. The suspensions were then centrifuged for 4 min in a Beckman microcentrifuge and the cellular extracts (supernatant) removed. Separation of a cytosol fraction or purification by affinity column was not done. The TK assay used was similar to that reported by Lee and Cheng [20]. The assay mixture consisted of: 0.112 M Tris-HCl (pH 7.5), 1.7 mM adenosine triphosphate, 1.7 mM $MgCl_2$, 0.8 μCi/ml [^{14}C]thymidine (New England Nuclear, Boston, MA; specific activity 55 mCi/mmol), 97 μM thymidine, 0.1% bovine serum albumin, 3 mM phosphocreatine, 0.54 unit creatine phosphokinase, 10 mM dithiothreitol, and 10 mM NaF. The cellular extracts (10 μl) were added in duplicate to tubes containing the assay mixture (75 μl). After incubation for 1 h at 37°C, the reaction was spotted (50 μl) onto Whatman DE81 discs (2.3 cm). The discs which retained phosphorylated nucleosides were washed 3 times in 95% ethanol, dried and analyzed in a scintillation counter in counting fluid (5.0 ml). One unit of TK activity was equal to the conversion of 1 nmol thymidine into the phosphorylated form per min at 37°C under the stated assay conditions.

For the dCyt-deaminase assay, the cell pellets were resuspended in 3 volumes of cold buffer (25 mM Tris-HCl, pH 8, 1 mM EDTA and 2 mM dithiothreitol) and the enzyme extracts were prepared as described above for the TK assay. The amount of

deaminase activity in the cellular extracts was determined by a method analogous to that of Steuart and Burke [29]. The reaction mixture (0.1 ml), containing 1.87 mM Tris-HCl (pH 8), 60 μM 2'-deoxycytidine, 0.3 μCi/ml [^{14}C]2'-deoxycytidine (New England Nuclear, Boston, MA; specific activity 25.2 μCi/mmol), was incubated for 2 h at 37°C with 10 μl of cellular extract. Fifty μl of cold trichloroacetic acid (1.2 M) was then added and the reaction mixture (100 μl) was loaded onto a pasteur pipette (4 × 0.8 cm) containing Dowex 50 (H^+ form) resin which had been previously equilibrated with water. The short column was washed with distilled water (3 times 500 μl). The product, [^{14}C]2'-deoxyuridine eluted with the void volume (1.5 ml), while the labeled substrate remained bound to the resin. The eluent was mixed with Aquasol-2 (4.5 ml; New England Nuclear, Boston, MA) and the number of counts per minute (cpm) was determined in a Beckman (model 330) liquid scintillation counter. The reaction was linear up to 12 000 cpm. One unit of deaminase activity was defined as the amount of enzyme which catalyses the production of 1 nmol 2'-deoxyuridine from 2'-deoxycytidine in 1 h at 37°C.

Protein assay

Protein concentrations were determined by the method of Hartree [15].

Results

Antiviral activity of FMAU

FMAU inhibited the replication of SB-1 in CEF and CKC (Fig. 1), GA-5 in CKC (Fig. 2b) and HVT in CEF (Fig. 3) in a dose-related manner. In these experiments,

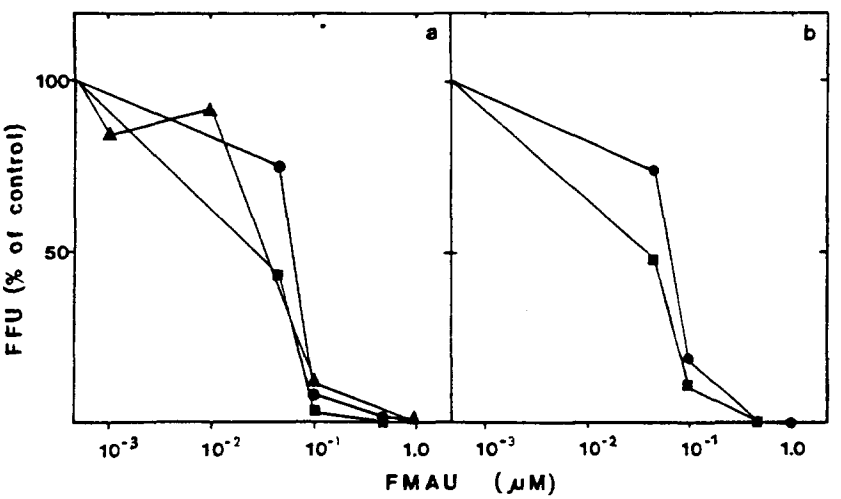

Fig. 1. Inhibition of SB-1 replication in chicken embryo fibroblasts (3 experiments, a) and chicken kidney cell (2 experiments, b) cultures by FMAU. Each set of identical symbols in a and b represents one experiment.

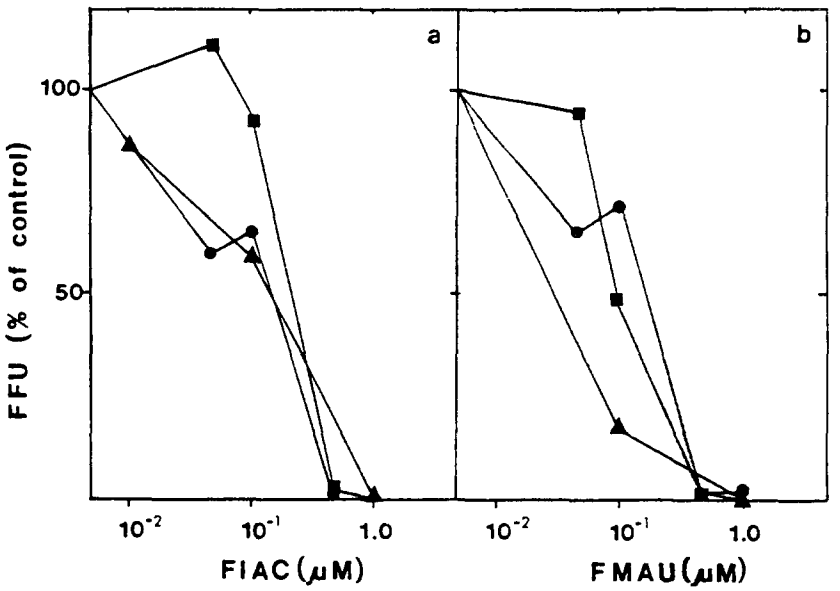

Fig. 2. Effect of FIAC (a) and FMAU (b) on virus replication of the oncogenic GA-5 strain of MDV in chick kidney cell cultures. Each set of identical symbols in a and b represents one experiment.

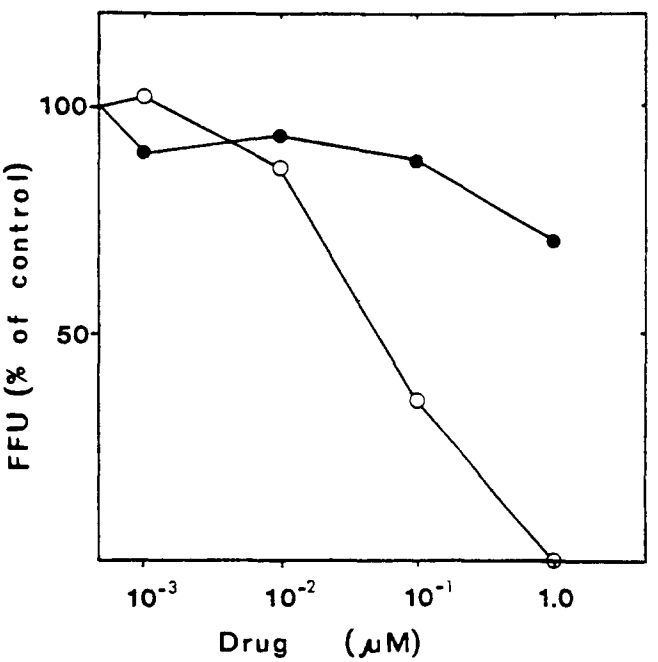

Fig. 3. Inhibition of HVT virus replication in chicken embryo fibroblast cultures by FMAU (○) and by FIAC (●).

0.5–1 μM FMAU significantly inhibited the replication of SB-1, GA-5, and HVT. The addition of FMAU 24 h after infection also inhibited viral replication with a dose–response curve identical to that seen when the drug was added prior to virus infection (data not shown). MDV infection of susceptible spleen cells was also prevented by 1.0 μM FMAU (Table 1).

Antiviral activity of FIAC and its metabolite FIAU

Whereas 1 μM of FIAC completely inhibited the replication of GA-5 and SB-1 in CKC cultures (Figs. 2a and 4), no antiviral effect was noted in CEF cells infected with SB-1 (Fig. 4). Similarly, HVT replication was not influenced by FIAC in CEF (Fig. 3). GA-5, an oncogenic strain of MDV, does not multiply efficiently in CEF and, therefore, could not be tested in these cells. Interestingly, FIAC was also unable to prevent in vitro infection of spleen cell cultures with MDV (Table 1). The cell-specific activity of FIAC suggested that CEF and lymphocytes lack dCyt-deaminase. The metabolite of FIAC, FIAU, in which the amino group of FIAC has been replaced by a hydroxy group, was used to investigate its antiviral activity against SB-1 in CEF and CKC. Viral replication was completely inhibited with 0.5 μM FIAU in both CEF and CKC (Table 2). FIAU was significantly more active than FIAC in CKC (50% effective dose 0.059 μM and 0.15 μM, respectively). When 400 μM THU was used to inhibit the dCyt-deaminase activity in CEF and CKC infected with SB-1, it abolished the antiviral activity of FIAC only in CKC and as expected did not alter the efficacy of FIAU (Table 2).

Anti-tumor activity of FMAU

FMAU was studied for its effect on two MD lymphoblastoid cell lines because it was effective in preventing the MDV infection in lymphocyte cultures (Table 1). FMAU did not inhibit the growth of CU-12 or CU-36; both cell lines replicated equally well in the presence or absence of 1 μM FMAU. Likewise, the expression of viral antigens was not inhibited in the cell line with high levels (CU-36) and low levels (CU-12) of antigen expression (data not shown). The absence of an inhibitory effect by FMAU was not caused by the selection of a drug-resistant variant of MDV. Virus could be isolated from CU-36 cultivated in the presence of FMAU for 24 days equally

TABLE 1

Inhibition of in vitro infection of lymphocytes with Marek's disease virus in the presence of the antiviral drugs FMAU and FIAC

Treatment		MDV-positive cells/ml 48 h p.i.[a]	
Drug	Concentration (μM)	1st pass.	2nd pass.
None	–	140	310
FIAC	1.0	170	271
FMAU	1.0	0	0

[a] Parallel cultures of treated but uninfected cells remained negative in all cases.

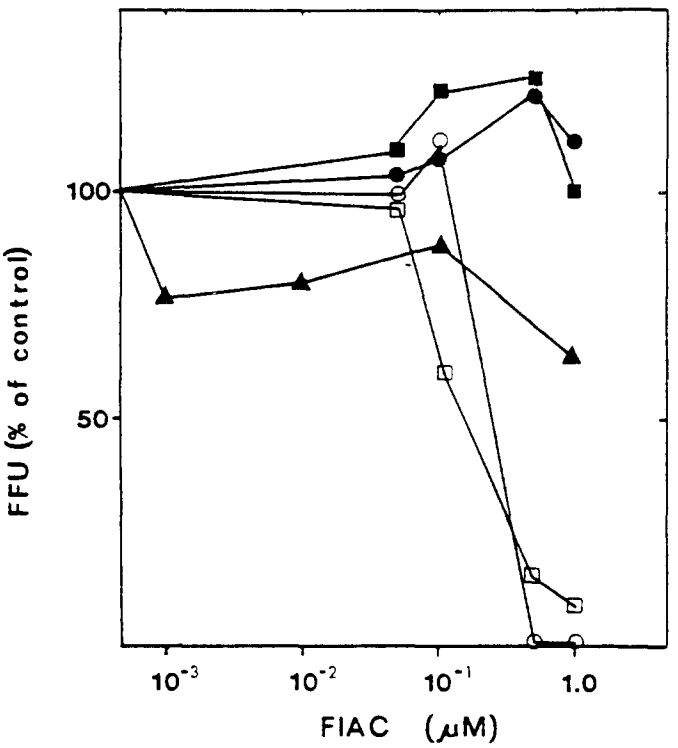

Fig. 4. Inhibition of SB-1 virus replication by FIAC in chicken kidney cell cultures (○,□, 2 experiments), and in chicken embryo fibroblasts (●,■,▲, 3 experiments). Each set of identical symbols represents one experiment.

TABLE 2

Competitive inhibition of cytosine nucleoside deaminase by 3,4,5,6-tetrahydrouridine (THU) in CKC cultures infected with SB-1

Drug	Concentration (μM)	FFU as % of control cultures[a]			
		CEF		CKC	
		-	THU[b]	-	THU[b]
FIAC	0.01	98	93	100	100
	0.1	83	98	90	108
	0.5	93	104	12	83
	1.0	105	114	0	80
FIAU	0.01	103	110	102	90
	0.1	4	5	24	16
	0.5	0	0	0	0
	1.0	0	0	0	0

[a] Average numbers of foci in the absence of FIAC, FIAU and THU were 90 in CEF and 30 in CKC. Data are from 3 replicate cultures per treatment. THU alone did not alter the number of foci.

[b] THU was added at a final concentration of 400 μM.

well as from the control line (44 and 54 FFU/2 × 10^6 cells, respectively). Moreover, the addition of 1 μM FMAU inhibited the isolation of virus irrespectively of the culture history.

Enzyme levels

The levels of dCyt-deaminase and TK in virus-infected CEF and CKC cultures were measured (Table 3). High levels of dCyt-deaminase were noted in uninfected or SB-1-infected CKC. In contrast, very low levels of this enzyme were noted in SB-1-infected or uninfected CEF cultures. Control cultures of uninfected HEp-2, Vero and rabbit kidney cells prepared in the same way had high, intermediate and low levels of the enzyme, respectively. The level of dCyt-deaminase reported here for HEp-2 cells was similar to those found by previous investigators [11]. It is noteworthy that the levels of dCyt-deaminase in CKC dropped significantly after infection with the SB-1 strain of MDV.

Since FIAC, FMAU and their metabolites are activated by viral-induced TK of herpes simplex virus [8,10], it was of interest to determine whether MDV and HVT also induce a TK in CKC cultures. The level of TK produced in JM-16-infected CKC was 1.5-fold higher than in uninfected cells, while HVT-infected CKC cultures had TK levels 13.5 times those of uninfected cells. SB-1-infected CKC had slightly higher TK levels than uninfected cells. However, the levels of TK activity were significantly lower for HVT, JM-16 and SB-1 than that of uninfected HEp-2 and Vero cells, or HeLa (Bu25) cells infected with HSV-1 (Table 3). Of the enzymes produced in the avian cell culture infected with virus, HVT produced the highest level of TK.

TABLE 3

Levels of 2'-deoxycytidine (dCyt)-deaminase and thymidine kinase (TK) in uninfected and virus-infected cells

Cells	Virus infection (strain)	Units of enzyme/mg protein[a]	
		dCyt-deaminase	TK
CEF	-	<0.05	ND[b]
CKC	-	27.2	0.011
CEF	MDV (SB-1)	<0.05	ND
CKC	MDV (SB-1)	8.1	0.018
CKC	MDV (JM-16)	ND	0.032
CKC	HVT (FC-126)	ND	0.15
HEp-2	-	257.0	1.23
Vero	-	6.3	0.79
Rabbit kidney	-	0.47	ND
HeLa (Bu25)[c]	-	ND	$<3 \times 10^{-4}$
HeLa (Bu25)	HSV-1 (F)	ND	1.19

[a] Variation between duplicate assays was 4%.

[b] ND = not determined.

[c] TK-deficient.

Discussion

An intensive search for selective anti-herpesvirus drugs has resulted in the development of a new group of nucleoside analogs [31], some of which have shown promise as antiviral and antitumor drugs [1,13,27,30]. MD provides an important model to study new antiviral drugs for several reasons. First, MDV can replicate in vitro not only in CKC, but also in bursa-derived lymphocytes, which are the principal target cells for the lytic infection in vivo. Second, several MD-derived lymphoblastoid cell lines have been established from tumors, which makes it possible to investigate antitumor activity. Third, MDV can induce atherosclerotic lesions in chickens, comparable to those seen in human beings [12]. It might, therefore, offer a model to investigate the effect of these drugs on virally induced atherosclerosis.

So far, only two other (nonnucleoside) antiviral drugs, phosphonoacetate and phosphonoformate, have been shown to inhibit the replication of MDV and HVT in cell culture [19,24]. This antiviral activity is probably due to the inhibition of the herpesvirus induced DNA-polymerase [19].

Our results indicate that FIAC requires dCyt-deaminase activity in some avian cells in order to exhibit antiviral activity. Other reports have shown that deamination of FIAC or its 5'-monophosphate is not essential for the activation of this drug in HSV-infected cells [10] (Schinazi, unpublished data). CEF lack dCyt-deaminase which is necessary to convert FIAC to FIAU. The antiviral activity of FIAC in CKC but not in CEF and the antiviral activity of FIAU in CKC and CEF, combined with the inhibition of dCyt-deaminase by THU in CKC, confirms the interpretation that the conversion of FIAC to FIAU is an important first step in order for FIAC to exert its anti-MDV and anti-HVT activity. For these reasons, it seems unlikely that the lack of antiviral activity of FIAC in CEF is caused by other characteristics of the cell type than the absence of dCyt-deaminase. The results point also to the importance of using an in vitro system that incorporates the in vivo target cell, as illustrated by the lack of activity of FIAC in lymphocyte cultures. A faulty conclusion about the potential efficacy of FIAC in vivo might have followed in vitro studies on MDV infection in CKC. Although definitive proof was not obtained, it seems plausible that the reason for the lack of activity of FIAC in spleen cell cultures was an absence of dCyt-deaminase in lymphocytes.

Although FMAU is reported to have activity against herpesvirus-induced lymphoblastoid cell lines [1], we were unable to detect any influence of the drug on the replication of the two cell lines studied. The lack of effect was not caused by the presence of a drug-resistant virus mutant. It is not yet determined if the MDV genome is present as episomal DNA or as completely or partly integrated DNA or as a combination of both [22]. It is evident that the maintenance of the viral genome in the transformed cell is not dependent on virally induced enzymes which would activate FMAU.

The studies on the induction of TK by HVT, SB-1 and JM-16 confirmed and extended the findings of Kit et al. [16,17] who reported that HVT induces a TK. It appears that the oncogenic JM-16 strain of MDV induces a TK albeit at lower levels than HVT. This might be a quantitative rather than a qualitative difference, because

HVT replicates faster and to higher titers than JM-16. It was not feasible to standardize the samples for virus content because MDV is more strictly cell-associated than HVT and the method of cell preparation caused considerable cell damage. It appears that SB-1 does not induce significant levels of TK and further studies are needed to confirm this finding. The presence of significant levels of TK in both infected and uninfected CKC suggests that sufficient TK is present in these cells to phosphorylate the fluoronucleoside to active metabolites.

In conclusion, FMAU and FIAU are potent inhibitors of the replication of MDV and HVT. In addition, the antiviral activity of FIAC on these viruses requires the presence of 2'-deoxycytidine deaminase in the avian host cell for activation.

Acknowledgements

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