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Inhibition of Epstein–Barr Virus Replication by a Novel L-Nucleoside, 2′-Fluoro-5-methyl-β-L-arabinofuranosyluracil

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ABSTRACT. A novel L-nucleoside analog, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU), was found to be a potent and selective inhibitor of Epstein–Barr virus (EBV) replication. The decrease in the amount of viral production was concentration dependent with a 90% inhibitory concentration of approximately 5 μM. Upon removal of the drug from treated cells, virus production resumed in 21 days. Metabolism studies indicated that L-FMAU could be converted to its mono-, di- and triphosphate metabolites in both EBV producing and non-producing cells. However, the amount of L-FMAU nucleotides formed was three times larger in EBV producing cells than in EBV non-producing cells. The mechanism of selectivity of L-FMAU against EBV does not appear to be due solely to the preferential phosphorylation of L-FMAU in EBV producing cells. The triphosphate of L-FMAU could not be utilized as a substrate by EBV DNA polymerase or the human DNA polymerases α, β, γ, or δ. Therefore, the incorporation of L-FMAU residues into viral DNA may not be the mechanism of antiviral activity. This compound appears to have a mechanism of action different from that of any other antiherpes virus nucleoside analogs. In addition, L-FMAU has very low cytotoxicity with 50% inhibition of cell growth occurring at a concentration of 1 mM. Given the potent inhibitory activity of this compound against EBV and its inability to be incorporated into cellular DNA, L-FMAU analogs should be explored as a new class of anti-EBV agents. BIOCHEM PHARMACOL 51;7:941–947, 1996.

KEY WORDS. L-nucleoside; L-FMAU; EBV; metabolism; DNA incorporation

EBV‖ is a major pathogen associated with infectious diseases as well as malignancies in humans [1–3]. Recently, a new virus that may be related to EBV has been identified as a possible pathogen of Kaposi's sarcoma in AIDS patients [4]. Therefore, the development of drugs with the ability to selectively inhibit EBV replication could have a major impact on the treatment of these diseases. Currently, there are no clinically effective anti-EBV agents without undesirable side-effects. Several nucleoside analogs have been found to be effective against EBV replication in cell culture at non-cytotoxic concentrations. These include ACV [5], FIAU [6], and DHPG [7]. ACV has been used to treat acute infectious mononucleosis and other EBV-associated disorders with low efficacy [8]. FIAU was found to have lethal toxic effects upon long-term usage in a recent anti-HBV clinical trial [9]. DHPG is also used clinically for the treatment of cytomegalovirus and was found to have substantial bone marrow toxicity [10]. The toxicity of these nucleoside analogs is likely due to their incorporation into cellular chromosomal and/or mitochondrial DNA. Recently, this laboratory and others have demonstrated that several cytosine L-nucleosides have potent activity against HBV and HIV [11–14]. However, these compounds have no selective activity against EBV (unpublished data). In this article, we describe some properties of the first novel thymidine L-nucleoside to have potent anti-EBV activity.

MATERIALS AND METHODS

Chemicals

D-FMAU, L-FMAU (Fig. 1) and [2-^{14}C]D-FMAU were synthesized by C. K. Chu, Department of Chemistry, University of Georgia. [*methyl*-^{3}H(N)]L-FMAU (76 Ci/mmol) was purchased from Moravek Biochemicals, Inc., Brea, CA. Both L-FMAU and [^{3}H]L-FMAU were purified further by HPLC on an RP-C18 column (Alltech Associates, Inc., Deerfield, IL) eluted with 20 mM KH_2PO_4, pH 5.5. DHPG

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‖ *Abbreviations:* EBV, Epstein–Barr virus; L-FMAU, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil; D-FMAU, 2′-fluoro-5-methyl-β-D-arabinofuranosyluracil; FIAU, 2′-fluoro-5-iodo-β-D-arabinofuranosyluracil; ACV, acyclovir; DHPG, ganciclovir; PCA, perchloric acid; pol α, DNA polymerase α; pol β, DNA polymerase β; γ, DNA polymerase γ; pol δ, DNA polymerase δ; pol ϵ, DNA polymerase ϵ; HBV, hepatitis B virus; and HIV, human immunodeficiency virus.

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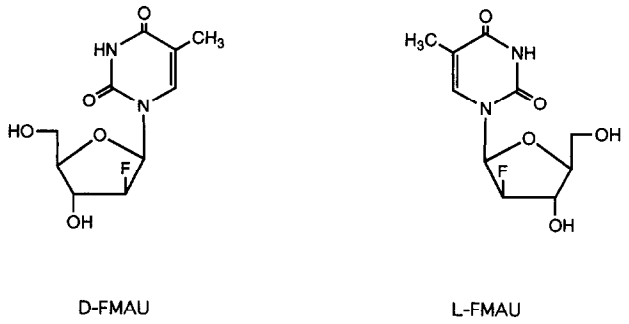

FIG. 1. Structures of D-FMAU and L-FMAU.

was a gift from Syntex, Inc., Palo Alto, CA; ACV was provided by Glaxo–Wellcome Laboratories, Triangle Park, NC; D-FMAUTP and L-FMAUTP were synthesized using a procedure described previously [15].

Cell Cultures

The EBV high virion-producing line (H1) and non-producing line (L5) [16] isolated from P3HR1 cell lines and another EBV non-producing line (Raji) were used in this study. All cells were cultured in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 100 μg/mL kanamycin at 37° in a humidified incubator containing 5% CO_2.

Exposure of H1 Cells to Drugs

H1 cells were treated with drugs as described previously [17]. The cells were seeded in 24-well plates at a density of 1×10^5 cells/well in 2 mL of fresh medium with or without drug and incubated at 37° for 5 days. Following the period of drug treatment, the cells were pelleted by centrifugation, and the amount of EBV DNA was determined. The medium containing virions was saved and evaluated for virion content.

The amount of EBV DNA was measured by the slot-blot method [18]. Briefly, the treated and non-treated H1 cells were lysed in 200 μL of 10 mM Tris–HCl (pH 7.5) using the freeze/thaw method. The cell lysate was treated with 10 μg/mL of RNase A at 37° for 30 min, and then with proteinase K (100 μg/mL) at 55° for 2 hr. The samples were spotted onto nylon membranes (Hybond-N, Amersham Corp., Arlington Heights, IL). Then a radiolabeled EBV *Eco* RI C fragment was used as a probe for DNA hybridization. The same membranes were reprobed with human *Alu* DNA after removing the EBV *Eco* RI C probe. The amount of EBV DNA in the treated and non-treated H1 cells was quantified by densitometry.

The virion titers secreted from treated and non-treated H1 cells were determined using a previously described bioassay [17]. Briefly, the virions were pelleted by centrifugation and used to infect 1×10^6 Raji cells for 48 hr. Since the level of EBV DNA polymerase activity in Raji cells post-superinfection is proportional to the number of virions added, the EBV-specific DNA polymerase activity induced in superinfected Raji cells was used as a parameter to estimate virion titer.

Cytotoxicity

The inhibition of H1 cell growth was evaluated as described previously [19]. H1 cells were seeded at 2×10^4 cells/mL and incubated with various concentrations of compounds for 4 days after which time the cell number was determined. IC_{50} was defined as the inhibitory concentration that caused a 50% reduction in cell number compared with the untreated control.

Metabolite Analysis

H1 and L5 cells (1×10^7 in 5 mL) were incubated with 10 μM [^{3}H]L-FMAU (14.5 μCi/mmol) for 24 hr. The cells were harvested by centrifugation at 600 *g* for 10 min, washed with cold PBS, and extracted with 100 μL of 0.5 N PCA. The acid-insoluble portion was separated by centrifugation for 10 min in a Beckman microfuge and washed three times with 200 μL of 0.5 N PCA before counting. Twenty-five microliters of 1 mM acyclovir triphosphate was added into the acid-soluble portion prior to separation to serve as an internal standard. Twelve microliters of 1 M $KHPO_4$ (pH 6.7) was added to the acid-soluble metabolites to serve as a buffer, and the mixture was neutralized with 5 N KOH. Five microliters of the neutralized metabolites was removed and counted for radioactivity, and the remaining fraction was analyzed by anion-exchange HPLC using a partisil 10 SAX column (Whatman, Inc., Clifton, NJ) and a step gradient that consisted of 30 mM potassium phosphate, pH 6.7, for 10 min, increased to 150 mM within 2 min, followed by 150 mM for 40 min and 300 mM potassium phosphate for 30 min. The amount of intracellular metabolites was normalized by the cell number and the internal standard.

Isopycnic Ultracentrifugation

H1 or L5 cells (1×10^7 in 5 mL) were incubated with 10 μM [^{3}H]L-FMAU (14.5 μCi/mmol) or 0.2 μM [^{14}C]D-FMAU (14.5 μCi/mmol) at 37° for 24 hr. The nucleic acids were isolated and analyzed by cesium sulfate isopycnic ultracentrifugation as described previously [20]. [*methyl*-^{14}C]Thymidine (51.7 mCi/mmol) was used as a positive control.

Enzyme Assay

Human pol α, pol β, pol γ and pol δ from K562 chronic myelogenous leukemia cells were purified as described previously [21, 22]. EBV DNA polymerase was partially purified from H1 cells [23]. Pol α and pol δ activities were assayed in 20 μL of 20 mM Tris–HCl buffer, pH 7.4, containing 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA,

100 μg/mL heat-inactivated bovine serum albumin, 20 μM dATP, dCTP, and dGTP, 2 μM [α-^{32}P]dTTP (1 μCi), and 150 μg/mL activated calf thymus DNA. The same assay was used for other DNA polymerases with the following modifications: pH 8.5 for pol β; pH 8.0 and 80 mM KCl for pol γ; and 100 mM $(NH_4)_2SO_4$ and pH 8.0 for EBV DNA polymerase. One to two units of DNA polymerase was used in each assay. The reactions were allowed to proceed for 30 min at 37° after which time a 15-μL aliquot was removed and spotted onto a Whatman DE-81 disc. The filters were washed with 0.3 M NaCl containing 0.5 mM EDTA and then fixed with ethanol. The incorporation of the radiolabeled substrate into the DNA chain was measured by liquid scintillation counting.

Chain Elongation Assays

Both D-FMAUTP (0.5–10 μM) and L-FMAUTP (1–100 μM) were analyzed for their incorporation into the 3′-terminus of the [5′-^{32}P]-14-mer annealed M13mp19 phage DNA primer–template complex. The sequence of the primer–template complex for DNA elongation assays was as follows:

M13mp19 phage DNA
3′-GGTCAAGTGCTGCAACATTTT
5′-CCAGTTCACGACGT

The primer was labeled with [γ-^{32}P]ATP (6000 Ci/mmol from Amersham) by T4 polynucleotide kinase [24].

Incorporation of the template-complementary nucleotide residue into the 3′-terminus of the primer was examined in 8 μL of a mixture containing the appropriate buffer solution as described above, enzyme, a 0.01 μM concentration of the primer–template complex and D-FMAUTP or L-FMAUTP. After 30 min of incubation, the reaction was stopped by adding 4.5 μL of formamide containing bromophenol dye and EDTA. The products were separated by 15% denaturated PAGE.

RESULTS

Effect of L-FMAU against EBV

The activity of L-FMAU against EBV production in H1 cells was examined. The results are shown in Table 1.

TABLE 1. Inhibitory effects of compounds on EBV

Drugs	IC_{50}* (μM)	EC_{90}* (μM)	SI†
DHPG	75 ± 6	5 ± 1	15
ACV	1000 ± 75	50 ± 8	20
L-FMAU	1000 ± 80	5 ± 0.8	200
D-FMAU	50 ± 5	0.1 ± 0.02	500

The IC_{50} was determined by exposing H1 cells to different concentrations of the selected compounds at 37° for 72 hr. The cells were then counted and compared with controls. The EC_{90} was determined from the amount of EBV DNA after 5 days of drug treatment, as described previously [17].

* Values are means ± SD of three individual experiments.

† SI = selective antiviral index, the ratio of IC_{50} to EC_{90}).

There was a concentration-dependent inhibition of EBV virion production with an EC_{90} value of 5 μM. L-FMAU did not inhibit H1 cell growth at 100 μM. The IC_{50} against H1 cell growth was estimated to be 1 mM. The antiviral potency of L-FMAU was stronger than ACV and comparable to DHPG but less than D-FMAU. The selective antiviral index (SI, the ratio of IC_{50} to EC_{90}) of L-FMAU was compared with those of compounds with known anti-EBV activity. They are in the order of D-FMAU > L-FMAU > ACV > DHPG in this culture system. The order of effectiveness could be different in other cell lines. The reversibility of anti-EBV activity of L-FMAU in comparison with that of ACV and DHPG was examined. H1 cells were exposed to two EC_{90} values of L-FMAU, ACV, and DHPG, respectively, for 5 days and then grown in drug-free medium for the time period indicated. Cells treated with L-FMAU, DHPG, and ACV could produce virus with titers of 70, 60, and 100%, respectively, in comparison with mock-treated cells on day 21 (Fig. 2). Thus, the antiviral action is either reversible or the concentration of these compounds is insufficient to inhibit all of the endogenous replicating EBV.

Metabolism of L-FMAU

The metabolism of L-FMAU in EBV producing cells and non-producing cells was examined. Two cell lines, H1 and L5, were isolated previously from HR1 cell lines. H1 cells can produce EBV and express viral early antigens including DNA polymerase and DNase, whereas L5 cells cannot produce EBV and do not express viral early antigens [16]. When both cell lines were incubated with 10 μM [^{3}H]L-FMAU or [^{14}C]D-FMAU for a period of 24 hr, the acid-soluble fraction, which includes intracellular nucleosides and nucleotides, and the acid-insoluble fraction, which includes DNA and RNA, were fractionated. The results are shown in Table 2. There was a substantial amount of D-FMAU in the acid-insoluble fraction, whereas around 3% of L-FMAU radioactivity was associated with the acid-insoluble fraction. The acid-soluble fractions of [^{3}H]L-FMAU-treated H1 and L5 cells were analyzed further by anion exchange HPLC. Three metabolites were identified as L-FMAUMP, L-FMAUDP and L-FMAUTP with retention times of 8, 20, and 34 min, respectively, in both cell lines (Fig. 3). L-FMAUMP was the major metabolite in both H1 and L5 cells. The amount of L-FMAUMP in H1 and L5 cell lines was estimated to be 140 and 60 pmol/10^6 cells, respectively. The L-FMAUTP and L-FMAUDP that accumulated were about the same in both cell lines (Fig. 3).

Incorporation of D-FMAUTP and L-FMAUTP into DNA

To determine whether L-FMAU could be incorporated into cellular or EBV DNA, H1 cells were exposed to equal anti-EBV concentrations of [^{3}H]L-FMAU (10 μM) or [^{14}C]D-FMAU (0.2 μM) for 24 hr. The nucleic acids were isolated and analyzed using cesium sulfate isopycnic ultracentrifu-

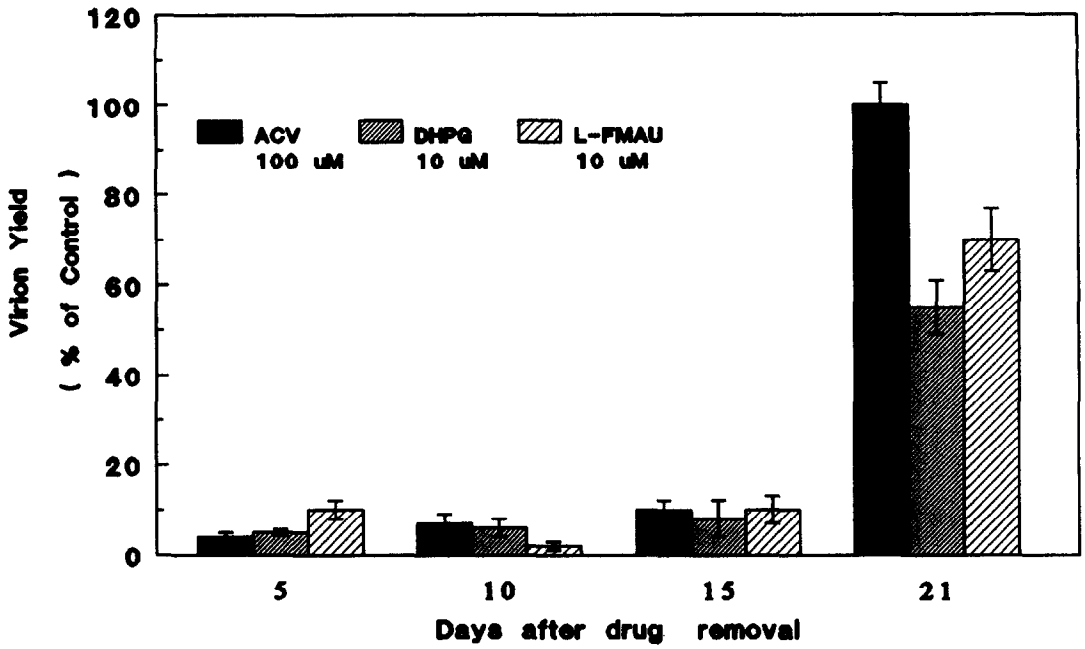

FIG. 2. Viral reversibility in H1 cells treated with L-FMAU. H1 cells were treated with ACV at 100 μM, DHPG at 10 μM, or L-FMAU at 10 μM for 5 days. The drugs were removed, and fresh drug-free medium was added. After removal of the drug, the amount of virion produced on days 5, 10, 15, and 21 was determined. One hundred percent of control was defined as the viral particles from equal amounts of untreated H1 cell culture medium. Data are the means (±SD) of three independent experiments with duplicate determinations performed in each experiment.

gation. Substantial amounts of [^{14}C]D-FMAU (higher than 10 pmol/10^6 cells) were incorporated into DNA (Fig. 4A); however, even with 50-fold excess drug treatment, insignificant incorporation of L-FMAU (less than 0.1 pmol/10^6 cells) was observed (Fig. 4B). No radioactivity of either compound was detected in RNA (Fig. 4).

Elongation of DNA Template by D-FMAUTP or L-FMAUTP

Incorporation of 5′-triphosphates of the compounds into DNA was analyzed by 15% denaturing PAGE. The results for EBV DNA polymerase (tracks 1 and 2), human pol α (tracks 3 and 4), pol δ (tracks 5 and 6), pol β (tracks 7 and 8), and pol γ (tracks 9 and 10) are illustrated in Fig. 5.

TABLE 2. Distribution of drug metabolites in H1 and L5 cells

Cells	Compound	% Distribution	
		Acid-soluble fraction	Acid-insoluble fraction
H1	L-FMAU	97.0 ± 3	3.0 ± 0.05
L5	L-FMAU	96.5 ± 8	3.5 ± 0.05
H1	D-FMAU	33.7 ± 0.3	66.3 ± 0.4
L5	D-FMAU	13.7 ± 0.5	86.3 ± 11

H1 and L5 cells (1×10^7 cells) were treated with either 10 μM [^{3}H]L-FMAU (14.5 μCi/mmol) or 0.2 μM [^{14}C]D-FMAU (14.5 μCi/mmol) for 24 hr at 37°. The concentration of drug used was two times the EC_{90}. Medium was removed, and acid-soluble and -insoluble fractions were extracted, as described in Materials and Methods.* Values are means ± SD of three individual experiments.

D-FMAUTP can be utilized as substrate by all DNA polymerases examined (tracks 1, 3, 5, 7, and 9). In contrast, L-FMAUTP could not be utilized by either EBV DNA polymerase (track 2) or human DNA polymerases (tracks 4, 6, 8, and 10) as substrate.

DISCUSSION

Nucleoside analogs continue to provide a rich source of antiviral agents that can suppress virus infection. A limiting factor in the clinical treatment of viral infectious diseases by nucleoside analogs is the toxicity, such as myelosuppression, resulting in anemia and neutropenia. The fact that viral enzymes are often quite similar to their cellular counterparts makes the development of effective non-toxic drugs against viral infections a rather challenging task. Recently, several L-nucleoside analogs were developed as a new class of compounds with potent inhibitory activity against HIV and HBV. No L-nucleoside analogs with anti-EBV activity have been described in the literature. L-FMAU, as demonstrated here, is the first L-thymidine analog found to have potent anti-EBV activity. This analog also has potent inhibitory activity against HBV but not against HSV-1 (unpublished data). The antiviral selectivity index of L-FMAU is better than that of ACV or DHPG.

D-FMAU, which has a better antiviral index than L-FMAU *in vitro*, was shown to exert toxicity in nonproliferating tissues upon long-term usage in patients. The mechanism of its delayed toxicity is likely the inhibition of mitochondrial function through its incorporation into mi-

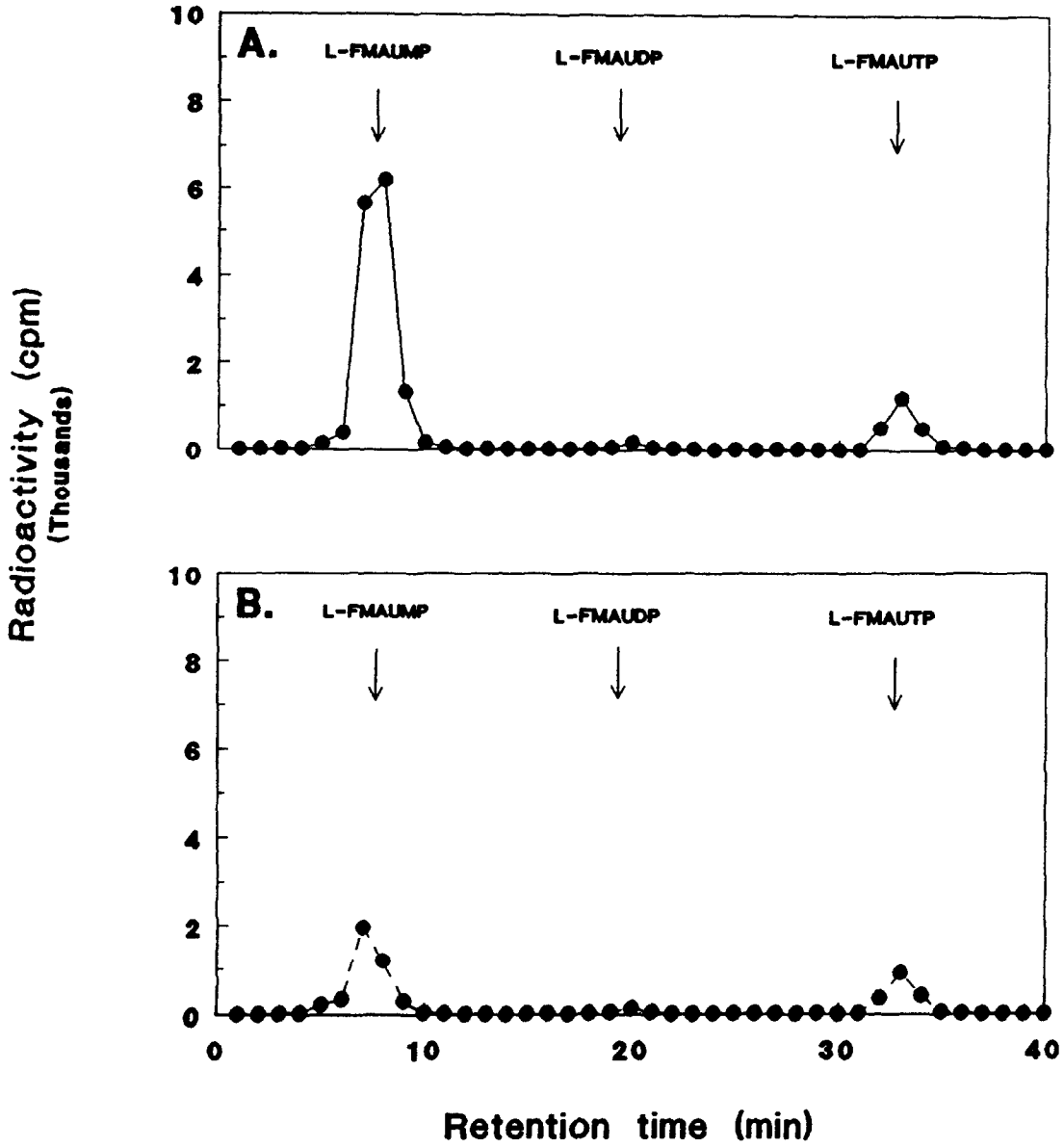

FIG. 3. Metabolism of L-FMAU in H1 and L5 cells. H1 (A) or L5 (B) cells (1×10^7 in 5 mL) were incubated with 10 μM [^{3}H]L-FMAU (14.5 μCi/mmol) for 24 hr. Both H1 and L5 showed three similar peaks with retention times at 8, 20, and 34 min, representing L-FMAUMP, L-FMAUDP and L-FMAUTP.

tochondrial DNA in nonproliferating affected tissues [25]. The treatment of EBV infection with L-FMAU may also require long-term usage due to the apparent reversibility of EBV infection. Therefore, it was important to examine whether L-FMAU could also be incorporated into cellular DNA. This question was addressed and will be discussed later.

The metabolic study of L-FMAU indicated that this compound could be converted to its mono-, di-, and triphosphate metabolites. The enzymes involved in this process are still under investigation. Given the differences in the formation of L-FMAUMP in H1 and L5 cells, it is possible that EBV-specified thymidine kinase in H1 cells could utilize L-FMAU as a substrate and could be responsible for the quantitative difference of L-FMAU nucleotides formed. Since L-FMAUMP could also be formed in L5 cells as well as in other non-EBV containing cells (unpublished results), human enzymes may also be capable of utilizing L-FMAU as a substrate. One interesting feature of L-FMAU metabolism is that the major metabolite is L-FMAUMP. This finding suggests that the intracellular conversion to L-FMAUDP from L-FMAUMP could be the rate-limiting step in the L-FMAU phosphorylation pathway.

The detection of 3% of [^{3}H]L-FMAU in the acid-insoluble fraction of cells raised the possibility that [^{3}H]L-FMAU could be incorporated into DNA. However, based on cesium sulfate gradient centrifugation, there was no detectable amount (less than 0.1 pmol/10^6 cells) of radioactivity in DNA. In contrast, D-FMAU was found to be incorporated into DNA in substantial amounts, even at one-fiftieth

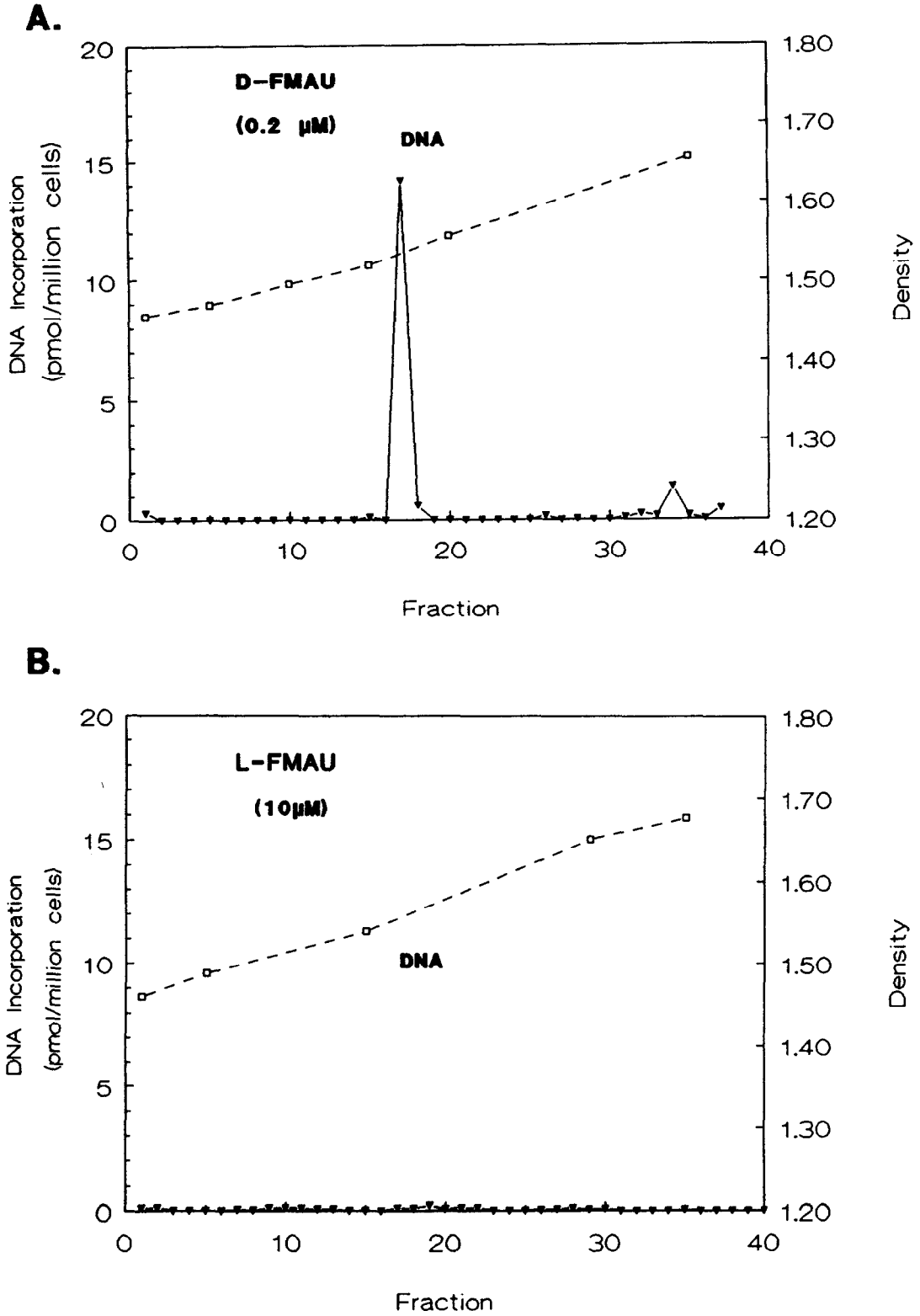

FIG. 4. Incorporation of [^{14}C]D-FMAU or [^{3}H]L-FMAU into DNA. H1 or L5 cells (1×10^7 in 5 mL) were incubated with 10 μM [^{3}H]L-FMAU (14.5 μCi/mmol) or 0.2 μM [^{14}C]D-FMAU (14.5 μCi/mmol) at 37° for 24 hr. The nucleic acids were isolated and subjected to isopycnic cesium sulfate gradient as described under Materials and Methods.

of the concentration of L-FMAU. Thus, the concern of toxicity manifested by D-FMAU in the clinic due to its incorporation into DNA may not be an issue for L-FMAU. The lack of incorporation of L-FMAU into cellular DNA could be due to the inability of human DNA polymerases to use L-FMAU as a substrate. It is also interesting that L-FMAUTP is not a substrate of EBV DNA polymerase. This finding suggests that the anti-EBV activity of L-FMAU

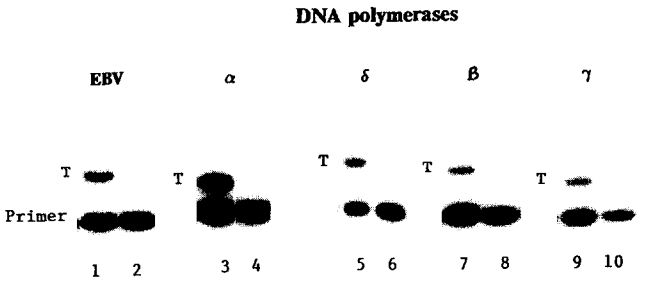

FIG. 5. Autoradiograph of the products of 5′-[^{32}P]primer elongation with 1 μM D-FMAUTP (tracks 1, 3, 5, 7, and 9) and 50 μM L-FMAUTP (tracks 2, 4, 6, 8, and 10) by EBV DNA polymerase (tracks 1 and 2) and human DNA polymerases α (tracks 3 and 4), δ (tracks 5 and 6), β (tracks 7 and 8), and γ (tracks 9 and 10). Experimental details are described in Materials and Methods.

may not be due to its incorporation into EBV DNA. All of the antiviral nucleoside analogs studied thus far with the exception of ribavirin exerted their antiviral action through their incorporation into viral DNA. Therefore, L-FMAU could have a very different mechanism of antiviral action from those antiviral compounds described. Detailed studies of its metabolism and mechanism of action are currently ongoing.

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