was again modified (*8*) to use iodoantipyrine, a freely diffusable, nonvolatile substance which has proven to be an effective tracer for quantitative measurements of LCBF (*3, 8, 10, 11, 15*).
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Epstein-Barr Virus: Inhibition of Replication by Three New Drugs

Abstract. *Epstein-Barr virus (EBV) is the cause of infectious mononucleosis and is associated with three human malignancies. Acyclovir [9-(2-hydroxyethoxymethyl)guanine], the first clinically useful drug effective against replication of EBV, is without effect against latent or persistent EBV infection. Three nucleoside analogs, E-5-(2-bromovinyl)-2'-deoxyuridine, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine, and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil are potent inhibitors of EBV replication in vitro. Moreover, in contrast to the reversibility of viral inhibition by Acyclovir, these three drugs have prolonged effects in suppressing viral replication even after the drugs are removed from persistently infected cell cultures.*

The hallmark of herpesviruses is their ability to cause latent and persistent as well as active infections. For the Epstein-Barr virus (EBV) there are cell systems in vitro that are counterparts of these three virologic states. In all of the diseases associated or caused by EBV infection—infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma and, as discovered recently, certain immunoblastic sarcomas that arise in allograft recipients (*1*)—the virus occurs in the pathologic cells in one, and often more than one, of these three states. In the past 2 years we have shown that Acyclovir (ACV) [9-(2-hydroxyethoxymethyl)guanine] (*2*) has potent effects against EBV infections in vitro when there is active replication of the virus (*3–5*). However, ACV has no effect in vitro on latent EBV infection, nor does the drug promise to be of much use in persistent low-level infection with EBV (*6*). Inasmuch as latent and persistent infection may be key elements of the oncogenic and chronic disease states associated with EBV, we started searching for other antiviral compounds active against EBV with the specific goal of identifying compounds that might have more prolonged effects than ACV.

Since all herpesviruses induce the formation of new viral DNA polymerases, we thought that drugs inhibitory to herpes simplex virus (HSV) might also inhibit EBV infection. We therefore selected several new nucleoside analogs for testing. Among the most promising were E-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) (*7–10*), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) (*11–13*), and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) (*11, 13*). All three compounds showed anti-EBV activity and had persistent effects in EBV-producing cell cultures.

We tested these compounds on EBV DNA replication in a virus-producing cell line (P3HR-1) and in Raji cells, a latently infected cell line, after superinfection with P3HR-1 virus. Cells were grown in RPMI 1640 medium (*14*). P3HR-1 cells spontaneously and continuously replicate large numbers of linear EBV genomes, synthesize early antigen (EA) and virus capsid antigen (VCA), and make virus particles. The level of cells showing spontaneous induction of virus replication fluctuates cyclically in 3 to 10 percent of the cells under our culture conditions. A steady-state mode of cell growth was established (*15*). Briefly, cells were seeded at a density of 4×10^5 to 6×10^5 cells per milliliter and counted daily until they reached 2×10^6 cells per milliliter. During this interval the cells were growing in a strictly exponential fashion.

The cells were harvested and resuspended in fresh RPMI 1640 medium containing the concentration of the drug to be tested, then incubated at 37°C for 5 days. The cells were harvested, and EBV genome copies per cell were determined by complementary RNA–DNA hybridization with an EBV-specific complementary RNA probe (*5, 14*). Table 1 shows that BVDU, FIAC, and FMAU,

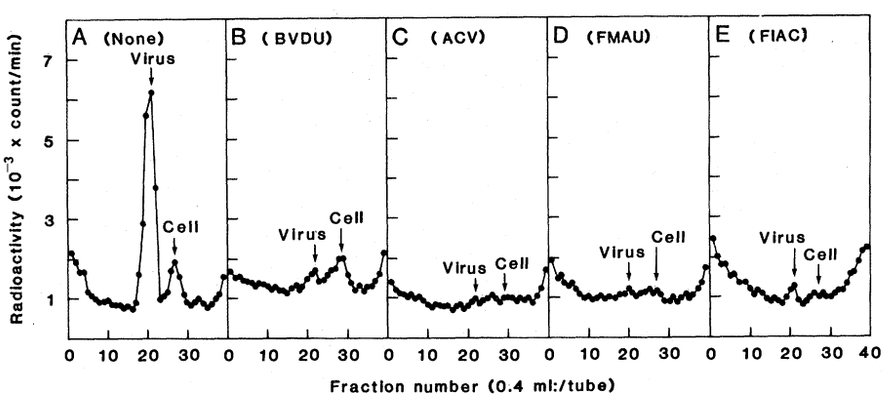

Fig. 1. Inhibition of EBV DNA replication by BVDU, FIAC, FMAU, and ACV in superinfected Raji cells. EBV DNA replication was determined by measuring the incorporation of ^{32}P into viral DNA and analyzing the DNA by cesium chloride density gradient centrifugation. The drug concentrations used were (A) no drug, (B) 20 μ*M* BVDU, (C) 100 μ*M* ACV, (D) 5 μ*M* FMAU, and (E) 10 μ*M* FIAC.

like ACV, were active against EBV. The concentrations chosen for each drug are known to be effective against herpes simplex virus and are at or below the median inhibitory dose (ID_{50}) for cytotoxicity (*12, 16*). All three drugs were at least as effective as ACV in reducing EBV genome numbers, and FMAU at a concentration of 10 μ*M* was more effective than ACV. On a molar basis, BVDU, FIAC, and FMAU were approximately 5, 10, and 50 times more potent than ACV, respectively.

We next investigated the drug effects in Raji cells superinfected with P3HR-1 virus, which results in shut-down of host functions and stimulation of viral DNA synthesis (*17*). A typical result is shown in Fig. 1. Viral DNA replication was greatly reduced in the presence of BVDU, ACV, FMAU, and FIAC, in contrast to the no-drug control in which a large quantity of EBV DNA was synthesized after superinfection. Thus, BVDU, FIAC, and FMAU clearly and effectively inhibited EBV DNA replication in two productive systems. EBV superinfection of Raji cells has a somewhat variable effect on cellular DNA synthesis, and no conclusions about the effects of the drugs on cellular DNA synthesis can be surmised in this system.

To assess whether the inhibitory effects of BVDU, FIAC, and FMAU were reversible, we treated P3HR-1 cells separately with each drug at the same effective doses (20 μ*M* BVDU, 10 μ*M* FIAC, and 5μ*M* FMAU) for 7 days and then placed them in drug-free medium and incubated them for an additional 10 days with ACV-treated cells as a control. Figure 2 shows that the inhibitory effect of ACV (100 μ*M*) was readily reversed upon removal of the drug. In contrast, the effects of BVDU, FIAC, and FMAU appeared not to be reversed; the effects persisted even after drug removal, and EBV genome numbers in FMAU-treated cells decreased further to 30 copies per cell after the drug release. The persistent effects of FIAC, FMAU, and BVDU were confirmed in experiments extending up to 58 days after removal of the drugs (data not shown).

The molecular basis for latent EBV infection in the nonvirus-producer Raji cells is the plasmid form of the EBV genome which is stably maintained in this cell line in a constant copy number (*17*). In the virus-producer line P3HR-1, the nonproductive fraction of the cell population also contains EBV plasmids (*3*). In neither type of cell are the EBV plasmids affected by ACV; in drug-treated, virus-producing cells the residual EBV DNA persists in this molecular form, and replication of linear genomes and virus resumes promptly when the drug is removed even after long periods of exposure (*3, 18*). The persistent effects of BVDU, FIAC, and FMAU suggest that these drugs, unlike ACV, are able to prevent the plasmid from recovering, at least for a time, as a functional template for viral replication.

Table 1. Effects of nucleoside analogs on Epstein-Barr virus genome replication in P3HR-1 cells. Exponentially growing cells were centrifuged and resuspended in fresh medium containing appropriate drugs and incubated at 37°C for 5 days. At the end of drug treatment the cells were harvested and the EBV genome copies per cell were determined.

Drug	Molarity (μ*M*)	EBV genome copies per cell*
FMAU	5	98
	10	48
BVDU	10	196
	20	87
FIAC	5	168
	10	106
ACV	100	98
None		396

*EBV genome copies were determined by complementary RNA-DNA hybridization with an EBV-specific complementary RNA probe (*15*). Results were the average of two determinations.

The three drugs, BVDU, FIAC, and FMAU, are potent inhibitors of EBV replication. That their prolonged effects could be a consequence of selective killing of the productive cells in P3HR-1 populations is suggested by the fact that during a 5-day treatment the cell viability decreased from 95 percent to approximately 45 percent. The viable cells could be rescued to resume normal growth, and viral genome numbers remained at a low level (30 copies per cell) for at least 45 days after rescue. The viability of BVDU-treated cells, however, remained the same as that of the untreated cells (95 percent). Alternatively, the persistent effects of the drugs could be due to the incorporation of these analogs or metabolic derivatives into the EBV plasmid DNA. However, since the EBV plasmids replicate every cell division (generation time, about 24 hours), incorporated analog would eventually be diluted out. Further studies may reveal the molecular and enzymatic mechanisms of these drugs against EBV.

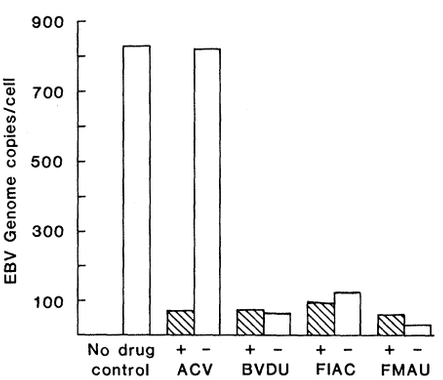

Fig. 2. Reversibility of effects of BVDU, FIAC, FMAU, and ACV on EBV DNA replication. P3HR-1 cells were treated separately with each drug for 7 days (hatched bars) and then released into drug-free medium and incubated for an additional 10 days (open bars). EBV genome copies per cell were determined as in Table 1. The drug concentrations were the same as in Fig. 1.

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