Trop. Med., 28 (Suppl.), 119—126, December, 1986

2′, 3′-Dideoxynucleoside Analogues: Development of Anti-Viral Therapy for HTLV-III/LAV Associated Diseases

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Introduction

The discovery of human T-lymphotropic virus type III (HTLV-III)/lymphadenopathy-associated virus (LAV) has intensified two lines of research which might potentially lead to control of the acquired immunodeficiency syndrome (AIDS) and a set of related diseases; 1) the development of a vaccine to protect persons who are not already infected with HTLV-III/LAV or are in a very early stage of infection, and 2) the development of agents active against HTLV-III/LAV which may have therapeutic effect in persons who are already infected.

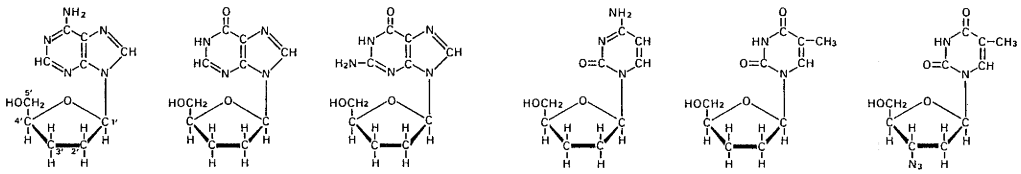
In this paper, we would like to describe several in vitro systems that we have developed to assess the activity of agents against HTLV-III/LAV; to discuss the in vitro antiviral activity of 2',3'-dideoxynucleoside analogues; and to report results of phase I clinical trial of one of the 2',3'-dideoxynucleoside derivatives, 3'-azido-3'-deoxythymidine (also called AZT or BW A509U). The structures of dideoxynucleoside analogues discussed in this paper are provided in Figure 1.

Development of Systems for Assessing Activity of Agents against HTLV-III/LAV

In the spring of 1984, after a new pathogenic retrovirus (HTLV-III/LAV) was proven to be the causative agent of AIDS and quantities of purified virus became available (Barre-Sinoussi et al, 1983; Popovic et al., 1984; Broder & Gallo, 1984; Wong-Staal & Gallo, 1985), we started to develop an anti-HTLV-III/LAV drug screening system in the Clinical Oncology Program of the National Cancer Institute.

We have now utilized a variety of approaches in the screening of drugs for potential usefulness in HTLV-III/LAV-related diseases (Table 1). In the initial step of screening we perform the HTLV-III/LAV cytopathic effect inhibition assay in which an immortalized T-cell line (ATH8) (vide infra) that is profoundly sensitive to the cytopathic effect of HTLV-III/LAV is used as target T-cells (Mitsuya et al., 1985a; Mitsuya & Broder, 1986). Clone ATH8 was generated by cloning a normal tetanus-toxoid specific T-cell line in the presence of human T-cell lymphotropic type I (HTLV-I) as previously described (Mitsuya et al., 1984a) and was selected for this study on the basis of its rapid growth [in the presence of interleukin 2 (IL-2)] and readily apparent sensitivity to the cytopathic effect of HTLV-III/LAV. Clone ATH8 bears several distinct copies of HTLV-I in its genome when assessed by Southern blot hybridization using a radiolabelled HTLV-I probe but nevertheless does not produce detectable amounts of HTLV-I gag proteins (Mitsuya, Reitz, and Broder; unpublished). It should be noted that several other HTLV-I-infected cell lines (e.g. MT2 and MT4) have been reported by Harada and Yamamoto to be sensitive to the cytopathic effect and infectivity of HTLV-III/LAV (Harada et al., 1985).

FIGURE 1. Structures of dideoxynucleosides discussed in this paper. The primed numbers (very left) refer to positions in the sugar moiety of nucleosides.



dideoxyadenosine dideoxyinosine dideoxyguanosine dideoxycytidine dideoxythymidine azido-thymidine

TABLE I. In Vitro Systems for Assessing Activity of Agents against HTLV-III/LAV.

1. Protection of antigen-specific helper/inducer T-cells against the cytopathic effect of HTLV-III$_B$, using normal and immortalized target cells.
2. Protection of target T-cells against the cytopathic effect of HTLV-III/RF-II (a variant of HTLV-III isolated from a Haitian patient), as an additional control for HTLV-III-isolate variability.
3. Inhibition of viral p24 expression in H9 cells, following exposure to HTLV-III$_B$.
4. Detection of HTLV-III DNA (using Southern blot hybridization) and RNA (using Northern blot hybridization) in target T-cells exposed to the virus in the presence and absence of the drug.
5. Effects on immune functions of normal T-cells *in vitro*.

When ATH8 cells are cultured in a test tube, these cells form a pellet at the bottom of the tube and the pellet size reflects the number of viable target T-cells. Under these conditions, the cytopathic effect is amenable to direct visual inspection. When exposed to HTLV-III/LAV, in the absence of protective drugs, the virus exerts a profound cytopathic effect on the target T-cells by day 4 in culture; by day 10, >98% of cells are killed by the virus and pellet of ATH8 cells is essentially completely destroyed. For example, as shown in Figure 2, ATH8 cells are almost completely killed by the virus by day 7 of culture following exposure to the virus (upper right). However, upon the addition of 2 μM 2',3'-dideoxycytidine (ddCyd) ATH8 cells are completely protected against the HTLV-III/LAV cytopathic effect and ATH8 cells are able to survive and grow (lower right) comparably to the virus-unexposed and drug-exposed control cells (upper left). It should be noted that the pellet size of ATH8 cells exposed only to ddCyd (lower left) is comparable to that of the virus-unexposed and drug-unexposed cells (upper left). The HTLV-III/LAV cytopathic effect inhibition assay thus permits the simultaneous assessment of potential anti-viral activity and toxicity of selective compounds. This assay is readily adaptable for mass screening (e.g. in microtiter plates). When nucleosides are tested in this system, various concentrations of the compounds are added to the culture following exposure to the virus. When possible neutralizing antibodies or certain compounds which slowly penetrate into cells, target cells are exposed to such agents prior to exposure to the virus and then continuously throughout the assay.

Anti-HTLV-III/LAV Activities of 2',3'-Dideoxynucleosides

To date, we have screened more than 250 different compounds including some 120 nucleoside derivatives for anti-HTLV-III/LAV activity. We have found that essentially every purine and pyrimidine nucleoside with a 2',3'-dideoxy-ribose moiety can block the infectivity and cytopathic effect of HTLV-III/LAV in vitro (Mitsuya & Broder, 1986). Figure 3 shows the potent protecitve effect of 2',3'-dideoxynulceosides on the survival and growth of ATH8 cells when exposed to HTLV-III/LAV. We found that concentrations of >10 μM 2',3'-dideoxyadenosine (ddAdo), 2',3'-dideoxyguanosine (ddGuo), 2',3'-dideoxyinosine (ddIno), as well as concentrations of >0.5 μM 2',3'-dideoxycytidine (ddCyd) completely protected ATH8 cells and enabled them to survive and grow. These compounds exhibited a strong

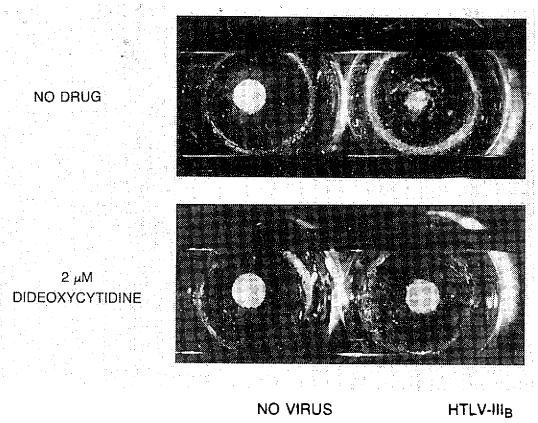


FIGURE 2. Cytopathic effect of HTLV-III/LAV on clone ATH8 and protection by 2',3'-dideoxycytidine. ATH8 cells ($2x10^5$) were exposed to $HTLV\text{-}III_B$ (3,000 virus particles per cell) and cultured in test tubes in the presence or absence of 2',3'-dideoxydideoxynucleosides. By day 7 of culture, ATH8 cells were almost completely killed by the virus. The cytopathic effect can be seen as a small disrupted pellet which contains debris of cells (upper right). In the presence of 2',3'-dideoxycytidine, ATH8 cells were completely protected and continued to grow, which can be seen as a large cell pellet (lower right) comparable to that of $HTLV\text{-}III_B$-unexposed, drug-unexposed ATH8 population (upper left). ATH8 cells exposed to only drug (lower left) formed a large pellet comparable to that of the control virus-unexposed, drug-unexposed cells (upper left).

anti-viral effect at doses that were lower by a factor of 10 to 20 than those that inhibited growth of the target cells when no virus was present. Under our experimental conditions, 2',3'-dideoxythymidine (ddThd) required relatively high concentrations to exert a protective effect, and unlike the other comparable dideoxynucleosides tested, its capacity to nullify the cytopathic effect of the virus was lost on day 10 of culture (data not shown).

The protective effects of 2',3'-dideoxynucleosides were confirmed in normal cloned helper/inducer T-cells (TM3) cocultured with irradiated $HTLV\text{-}III_B$-producing H9 cells ($H9/HTLV\text{-}III_B$) ($H9/HTLV\text{-}III_B$ cells serve as a source of infectious virions, and theoretically both cell-free and cell-associated virus transmission can occur). Moreover, the anti-HTLV-III/LAV activity of dideoxynucleosides was confirmed by their inhibitory effect on the expression of HTLV-III/LAV p24 gag protein in H9 cells.

In order to determine whether the 2',3'-dideoxyribose moiety was necessary for the anti-HTLV-III/LAV activity, we explored the capacity of five closely related adenosine congeners [2',3'-dideoxyadenosine, 2'-deoxyadenosine, 3'-deoxyadenosine (also called cordycepin), adenine arabinoside (ara-A), and 2',3',5'-trideoxyadenosine] to block the cytopathic effect of HTLV-III/LAV (Mitsuya & Broder, 1986). Results indicate that at least among these closely related adenosine congeners tested, only adenosine with a 2',3'-dideoxyribose moiety has an anti-HTLV-III/LAV activity.

It is worth noting that 2',3'-dideoxynucleosides and related compounds such as 3'-azido-3'-deoxythymidine are not new chemicals and pioneering studies on several of these compounds were initiated in the 1960's and 1970's, before human retroviruses were proved to exist (Robins & Robins, 1964; Horwitz et al., 1964; McGovern et al, 1966; Toji & Cohen, 1969; Lin & Prusoff, 1978).

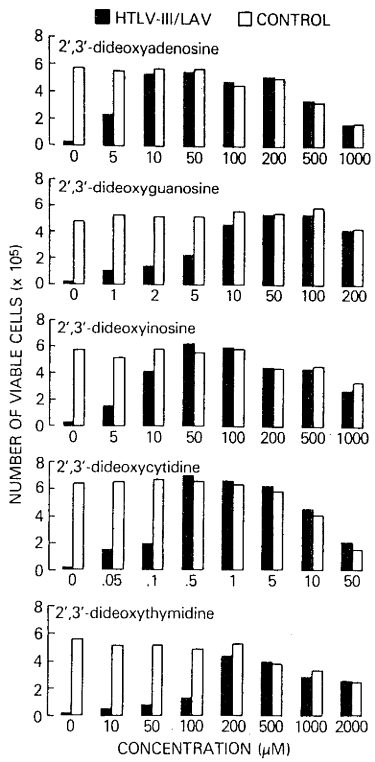


FIGURE 3. Inhibition of cytopathic effect of HTLV-III/LAV by 2',3'-dideoxynucleosides against ATH8 cells. ATH8 cells ($2x10^5$) were pre-exposed to polybrene, exposed to $HTLV\text{-}III_B$ (2,000 virus particles per cell) and cultured in test tubes (solid columns) in the presence or absence of various concentrations of 2',3'-dideoxyadenosine, -guanosine, -inosine, -cytidine, or -thymidine. Control cells (open columns) were similarly treated, but were not exposed to the virus. On day 5, total viable cells were counted. Reproduced by permission of the Proceedings Office of the National Academy of Sciences, USA (Mitsuya & Broder, 1986).

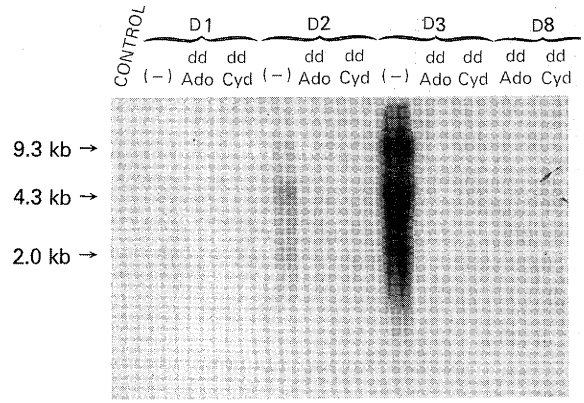


FIGURE 4. Lack of HTLV-III/LAV mRNA expression in susceptible T-cells exposed to the virus but protected by 2',3'-dideoxyadenosine or 2',3'-dideoxycytidine. $2x10^7$ ATH8 cells were treated with polybrene, exposed to HTLV-III_B (1,000 virus particles per cell), resuspended, and cultured in the presence or absence of 50 μM 2',3'-dideoxyadenosine or 2 μM 2',3'-dideoxycytidine in culture flasks. On days 1, 2, 3, and 8 following exposure to the virus, total RNA was extracted and subjected to Northern blot hybridization using radiolabelled HTLV-III/LAV antisense RNA. The 9.3 kb segment represnts HTLV-III/LAV genomic RNA; the 4.3 kb band env RNA; and the 2 kb tat-III and 3'-orf RNA species. Control denotes total RNA from ATH8 cells which were not exposed to the virus.

Inhibition of HTLV-III/LAV DNA Synthesis and RNA Expression in T-cells Exposed to the Virus.

In cells infected with HTLV-III/LAV, the virus can be detected as unintegrated and integrated viral DNA as well as viral mRNA (Hahn et al., 1984). Southern and Northern blot hybridization techniques are potentially useful in determination of the relative amounts of viral DNA and RNA of the virus-harboring cells and tissues and have enabled us to detect HTLV-III/LAV even when the virus is residing in the latent form.

We have asked if viral DNA and mRNA can be detected in susceptible T-cells (ATH8) when exposed to the virus but protected by putative drugs. In a typical experiment, ATH8 cells are exposed to HTLV-III/LAV and cultured in the presence or absence of selective drugs. High molecular DNA is extracted at various times and assayed for its content of viral DNA using a radiolabelled HTLV-III/LAV probe. In the absence of protective agents under our culture conditions, viral DNA is first detected by day 2, and a substantial amount of viral DNA is detected on the following day. In contrast, in DNA from ATH8 cells which have been completely protected by certain effective drugs, neither unintegrated nor integrated DNA is detected. We also ask whether viral mRNA is expressed in target T-cells exposed to the virus and cultured with or without protective agents. Figure 4 shows that ddAdo and ddCyd completely inhibited the HTLV-III/LAV mRNA expression in ATH8 cells exposed to the virus. In this experiment, ATH8 cells were exposed to HTLV-III_B (1,000 virions per cell), and RNA was extracted from ATH8 cells on days 1, 2, 3, and 8 of culture. Extracted RNA was then assayed for the content of viral mRNA by Northern blot hybridization using a radiolabelled HTLV-III/LAV antisense RNA probe. In the absence of dideoxynucleosides viral mRNA was detectable on day 2 and was present at a greater level on day 3. In contrast, when cultured in the presence of 50 μM ddAdo or 2 μM ddCyd, viral RNA could not be detected throughout the study. (Mitsuya, Jarrett, Matsukura, and Broder; unpublished).

This assay system allows us to assess the capacity of drugs to inhibit HTLV-III/LAV DNA synthesis and mRNA expression in T-cells exposed to the virus, and also perhaps allows us to assess the mechanism of the antiviral effects. Our working conclusion is that the dideoxynucleosides can inhibit HTLV-III/LAV at the point of reverse transcription (vide infra).

Mechanism(s) of Anti-Retroviral Activity of 2',3'-Dideoxynucleosides

2',3'-Dideoxynucleosides are nucleoside analogues in which both the 2'- and 3'-hydroxy (OH-) groups are replaced with a hydrogen. When the dideoxynucleosides are converted to a triphophate form, it is not possible to form normal 5'→3' phosphodiester linkages which are necessary for DNA elongation in the replication of virus from an RNA form to a DNA form. Indeed, we have shown that ddCyd is converted to a triphosphate by cellular enzymes (Cooney et al., 1986), and the triphosphate product is utilized by HTLV-III/LAV DNA polymerase (or reverse transcriptase; RT) and inhibits DNA synthesis mediated by purified HTLV-III/LAV RT. Certain dideoxynucleosides as a triphosphate form can thus act as DNA chain terminators in viral DNA synthesis although this need not be the only mechanism for anti-retroviral activity. It has been shown that the 5'-triphosphate products of ddAdo, ddGuo, ddCyd, and ddThd can rather easily inhibit mammalian DNA polymerases beta, gamma, as well as RT of animal retroviruses, but not mammalian DNA polymerase alpha (Waqar et al., 1984); and a similar observation has been made by us with HTLV-III/LAV (Mitsuya, Spigelman, Veronese, McCaffrey, Sarngadharan, and Broder; unpublished). DNA polymerase alpha is assumed to be the key DNA synthetic enzyme for DNA replication during cell growth, and it also has a role in DNA repair (Waqar et al., 1984; Miller & Chinault, 1982; Krokan et al., 1979). These four dideoxynuclesides have rather negligible short-term effects on the growth of cultured mammalian cells (Waqar et al., 1984). Indeed, at concentrations that suppressed the replication of the virus, virtually all dideoxynucleosides we tested failed to affect the growth and function of normal T-cells except at very high doses (Mitsuya & Broder, 1986). DdAdo and ddCyd have been selected for testing in humans with HTLV-III/LAV infection, and ddCyd is presently undergoing animal toxicity testing.

We believe 2',3'-dideoxynucleosides could potentially have an effect against any retrovirus. For example, we have recently found that two dideoxynucleosides, ddCyd and 3'-azido-3'-deoxythymidine (an azido derivative of ddThd), block the infectivity of HTLV-I against normal helper/inducer T-cells (Matsushita, Mitsuya, Reitz, and Broder; unpublished). We have also observed that 2',3'-dideoxynucleosides can block the replication of certain animal retroviruses (Dahlberg, Mitsuya, Broder, and Aaronson; unpublished). These properties, taken together, suggest that at least some dideoxynucleosides are attractive candidates for further development for treatment of human and veterinary retrovirus-related diseases.

In Vitro Administration of 3'-Azido-3'-deoxythymidine (AZT or BW A509U)

As noted above, we have found that some compounds which are chain terminators for DNA synthesis can act as potent inhibitors of retroviral replication. One such compound, 3'-azido-3'-deoxythymidine (also called AZT or BW A509U), has recently been studied in a Phase I trial (Yarchoan et al. 1986). AZT is a ddThd analogue in which the 3'-hydroxy group is replaced by an azido group (see Figure 1). At concentrations of 1 -3 uM, this drug inhibits the *in vitro* infectivity and cytopathic effect of HTLV-III/LAV in helper/inducer T-cells (Figure 5) (Mitsuya et al., 1985a). Based in part on these *in vitro* data, a trial of AZT in patients with AIDS and AIDS-related complex (ARC) was initiated at the National Cancer Institute and Duke University Medical Center in collaboration with Wellcome Research Laboratories. In a 6-week clinical trial 4 dose regimens of AZT were examined in 19 patients. The four dose regimens were A) 1 mg/kg intravenous infusion (IV), then 2 mg/kg peroral administration (PO) every 8 hr; B) 2.5 mg/kg IV, then 5 mg/kg PO every 8 hr; C) 2.5 mg/kg IV, then 5 mg/kg PO every 4 hr; and D) 5 mg/kg IV, then 10 mg/kg PO every 4 hr. Pharmacokinetic studies showed that peak levels of 1.5 to 2 uM were attained following a one hour infusion of 1 mg/kg or oral administration of 2 mg/kg and that the drug has a half-life of approximately 1 hr. Increased doses of the drug yielded proportionally increased peak

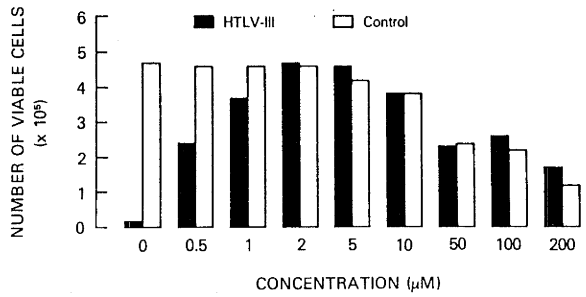


FIGURE 5. Inhibition of cytopathic effect of HTLV-III/LAV by 3'-azido-3'-deoxythymidine against ATH8 cells. ATH8 cells ($2x10^5$) were pre-exposed to polybrene, exposed to $HTLV\text{-}III_B$ (2,000 virus particles/cell), and cultured in culture tubes (solid columns) in the presence or absence of various concentrations of AZT. Control cells (open columns) were similarly treated, but were not exposed to the virus. On day 5, total viable cells were counted.

levels; for example, 5 mg/kg given intravenously over one hour yielded a peak plasma level of 6 to 10 uM and a level of 0.6 uM 4 hours after the start of the infusion. In addition, sampling of the cerebral spinal fluid (CSF) showed penetration of AZT. The drug was in general well tolerated during short term administration; side effects were : 1) 5 - 10% hematocrit drops in several patients, 2) headaches in approximately half the patients, 3) tremors, confusion, and neutropenia in one patient who was simultaneously receiving trimethoprim-sulfamethoxazole, and 4) decrease in the total white blood count in half of the patients on the highest two doses.

While the primary purpose of this study was to determine the safety and feasibility of administrating AZT to patients with AIDS and ARC over 6 weeks, the results in addition indicated that immunological and/or clinical responses were observed in some of the patients during this short-term administration. In particular, 15 of 19 patients had increases in numbers of their circulating helper/inducer T-lymphocytes, 6 previously anergic patients gave positive skin test during treatment, and the 1 patient in whom it was tested showed restoration of a virus-specific cytotoxic T-cell response. Furthermore, 2 patients showed a clearing of chronic fungal nailbed infections, 13 patients had weight gains of 2 kg or greater, and 6 patients had other clinical improvement. While it is likey that these changes were the result of taking AZT, it is hard in such a small uncontrolled study to separate out such contributing factors as spontaneous improvement or a placebo effect.

Although our findings suggest partial immunological reconstitution in certain patients given AZT, it must be stressed that the increases in numbers of helper/inducer T cells were often small and were most substantial during the first 2 weeks of intravenous therapy; we cannot be sure that starting with an oral course of therapy will yield similar results. Also, the decreases in helper/inducer T cells or total white blood cells in some patients on the highest dose tested suggest that this dose may be lymphotoxic. Recent observations suggest that the accumulation of phophorylated AZT within cells causes a substantial depression of thymidine triphophate (Balzarini and Broder, unpublished), and this may contribute to the toxicity at high doses.

These initial results with AZT thus suggest that it can, at least for a 6 week period, induce immunologic improvemnt in patients with HTLV-III/LAV disease, and that in some patients, it can yield other clinical benefits. A randomized double-blind placebo controlled study of orally administered AZT is presently being organized by Burroughs Wellcome Co. in several medical centers thoughout

the United States to further evaluate this drug, specifically to determine whether it can be tolerated for 6 months, whether it can yield immunologic benefits over this time period, and whether it can reduce the morbidity and mortality of patiens with AIDS. Such controlled trials will offer the best way of resolving the role of AZT in the therapy of patients with AIDS.

Conclusion

In the last two years, more than a dozen drugs have been identified to have anti-HTLV-III/LAV activity (see Yarchoan et al., 1985 for review; Mitsuya et al., 1984b; McCormick et al., 1984; Rosenbaum et al., 1985; Mitsuya et al., 1985b; Broder et al., 1985; Sandstrom et al., 1985; Ho et al., 1985). While there is clearly a long way to go before an effective therapy is developed for HTLV-III/LAV diseases, we hope we have gotten off to a good start. At least several efficient screening systems for agents active against HTLV-III/LAV have been reported and several drugs identified in these systems have been tested in patients with HTLV-III/LAV infection. Also, we are gaining experience in the monitoring of patients to identify clinical changes which may occur as a result of antiviral therapy in HTLV-III/LAV diseases. We believe that as we learn more about HTLV-III/LAV, we can more rationally develop therapeutic strategies which might have considerable value in attaking this disease.

Acknowledgements

We thank Drs. Ruth Jarrett, Makoto Matsukura, Shuzo Matsushita, and Marvin Reitz for their help and discussions in these studies and Drs. Sandra Lehman and David Barry for providing 3'-azido-3'-deoxythymidine. We also thank Dr. Robert Gallo for providing HTLV-III/LAV virus and H9 cell line.

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